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Vitamin D Receptor and Enzyme Expression in Dorsal Root Ganglia of Adult Female Rats: Modulation by Ovarian Hormones

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Abstract

Vitamin D insufficiency impacts sensory processes including pain and proprioception, but little is known regarding vitamin D signaling in adult sensory neurons. We analyzed female rat dorsal root ganglia (DRG) for vitamin receptor (VDR) and the vitamin D metabolizing enzymes CYP27B1 and CYP24. Western blots and immunofluorescence revealed the presence of these proteins in sensory neurons. Nuclear VDR immunoreactivity was present within nearly all neurons, while cytoplasmic VDR was found preferentially in unmyelinated calcitonin gene-related peptide (CGRP)-positive neurons, colocalizing with CYP27B1 and CYP24. These data suggest that $1,25(OH)_2D3$ may affect sensory neurons through nuclear or extranuclear signaling pathways. In addition, local vitamin D metabolite concentrations in unmyelinated sensory neurons may be controlled through expression of CYP27B1 and CYP24. Because vitamin D deficiency appears to exacerbate some peri-menopausal pain syndromes, we assessed the effect of ovariectomy on vitamin D-related proteins. Two weeks following ovariectomy, total VDR expression in DRG dropped significantly, owing to a slight decrease in the percentage of total neurons expressing nuclear VDR and a large drop in unmyelinated CGRP-positive neurons expressing cytoplasmic VDR. Total CYP27B1 expression dropped significantly, predominantly due to decreased expression within unmyelinated CGRP-positive neurons. CYP24 expression remained unchanged. Therefore, unmyelinated CGRP-positive neurons appear to have a distinct vitamin D phenotype with hormonally-regulated ligand and receptor levels. These findings imply that vitamin D signaling may play a specialized role in a neural cell population that is primarily nociceptive.

Keywords

1,25-dihydroxyvitamin D3; reproductive hormones; pain; sensory; CYP27B1; CYP24

Introduction

Vitamin D metabolites have widespread roles in human health. In addition to well known actions on calcium homeostasis and bone remodeling, vitamin D has been linked to immunity, cardiovascular disease, and cancer (Holick and Chen, 2008). Accumulating evidence supports a role for vitamin D in certain types of pain. For example, vitamin D deficiency is associated with chronic musculoskeletal pain and acroparaesthesia (numbness,

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burning, or tingling in the extremities) (Glerup and Eriksen, 1999; Gloth *et al.*, 1991; Masood *et al.*, 1989; Plotnikoff and Quigley, 2003). Over 85% of patients who report chronic musculoskeletal pain have levels of serum 25-hydroxyvitamin D_3 ($25OHD_3$) that are less than 20ng/ml (50nmol/L) (Benson *et al.*, 2006; Macfarlane *et al.*, 2005; Plotnikoff and Quigley, 2003), which is relieved by vitamin D supplementation (de Torrente de la Jara *et al.*, 2006; Glerup and Eriksen, 1999; Gloth *et al.*, 1991). However, the mechanism by which vitamin D deficiency leads to altered sensation is unknown.

Vitamin D_3 is derived from diet or exposure to sunlight, converted in the liver to the main circulating form, 25-hydroxyvitamin D_3 ($25OHD_3$), which is further transformed by 25-hydroxyvitamin D 1α-hydroxylase/cytochrome P450, family 27, subfamily B, polypeptide 1 (CYP27B1) into 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), which is the most well understood active metabolite. Conversion of $25OHD_3$ into $1,25(OH)_2D_3$ occurs largely in kidney, but other cell types express CYP27B1 and can locally convert circulating precursor to active hormone (Jones, 2007). $1,25(OH)_2D_3$ binds to vitamin D receptors (VDRs), which are spatially dynamic and may be localized within the nucleus where they influence gene transcription, or within the cytoplasm and membrane where they modulate ion flux and the generation of second messengers (Mizwicki and Norman, 2009). Hydroxylation of $1,25(OH)_2D_3$ to form 1,24,25-trihydroxyvitamin D_3 by the widely expressed enzyme, 25-hydroxyvitamin D 24-hydroxylase/cytochrome P450, family 24, subfamily A, polypeptide 1 (CYP24), is an initial step in the pathway of $1,25(OH)_2D_3$ degradation, but CYP24 also produces other vitamin D metabolites, whose actions are just beginning to be understood (Omdahl *et al.*, 2003; Sakaki *et al.*, 2005). The extent to which these vitamin D-related proteins are present within adult sensory neurons is unknown.

A number of factors can influence VDR signaling, including ovarian hormones. For instance, VDR expression is generally up-regulated by 17β-estradiol (Gilad *et al.*, 2005). Further, estrogens can increase $1,25(OH)_2D_3$ concentrations locally by increasing CYP27B1 and decreasing CYP24 levels (Lechner *et al.*, 2006). In addition, progesterone has been reported to enhance estrogen-induced CYP27B1 expression (Tanaka *et al.*, 1978). While it is not known whether ovarian hormones and vitamin D signaling mechanisms interact in sensory nerve pathways, peripheral sensory neurons do express estrogen and progesterone receptors (Chan *et al.*, 2000; Sohrabji *et al.*, 1994), and anecdotal evidence suggests that vitamin D and ovarian hormones may interact to influence pain sensation. For example, musculoskeletal pain is more prevalent in females and frequently exacerbated by menopause or pharmacological estrogen suppression (Andersson *et al.*, 1993; Bergman *et al.*, 2001; Croft *et al.*, 1993; Dugan *et al.*, 2006; Felson and Cummings, 2005; Friedman *et al.*, 1993; Garreau *et al.*, 2006; Greendale *et al.*, 1998; Morales *et al.*, 2006; Mouridsen, 2006; White *et al.*, 1999; Wolfe *et al.*, 1995). Moreover, musculoskeletal pain induced by hypoestrogenemia is inversely correlated with serum $25(OH)D_3$ levels and can be partially ameliorated by high-dose vitamin D_3 therapy (Khan *et al.*, 2009; Waltman *et al.*, 2009). To assess the potential for vitamin D signaling in peripheral sensory neurons and the possible influence of reproductive hormones, we examined expression patterns of VDR, CYP27B1, and CYP24 in adult dorsal root ganglia (DRG) in intact and ovariectomized female rats.

Materials and Methods

Experimental preparations

Five female Sprague Dawley (Harlan Laboratories, Indianapolis, IN) rats at six weeks of age were ovariectomized by bilateral hind flank incision following anesthesia with 60mg/kg ketamine (Pfizer, New York, New York), 0.4mg/kg atropine (Baxter, Deerfield, IL), 8mg/kg xylazine (Lloyd Laboratories, Shenandoah, Iowa). Four age-matched female rats were allowed to cycle normally and estrous cycle stage was tracked daily by vaginal lavage with

an eyedropper and 0.9% saline (Becker *et al.*, 2005). After two weeks, all rats were euthanized by i.p. injection of sodium pentobarbital (150mg/kg, Ovation) followed by decapitation; cycling rats cycled normally during this two week period and were euthanized at estrus, as determined by an opaque vaginal smear consisting predominantly of cornified epithelial cells. All rats were exposed to a 14hr light/10hr dark cycle and sacrificed 4–6 hr into the light cycle. All procedures were reviewed by the University of Kansas Medical Center Institutional Animal Care and Use Committee and conformed to all local and federal guidelines.

DRGs from C3-S1 were removed and all left ganglia from each animal were pooled and stored in RNAlater (Ambion, Austin, Texas) at −20°C for western blot protein analysis. The right DRGs were embedded in tissue chilled freezing medium (Electron Microscopy Sciences, Hatfield, PA), snap frozen, and stored at −80°C for immunofluorescence studies.

Antibody Characterization

In preliminary experiments several anti-VDR antibodies were tested by immunohistochemistry on rat DRG sections. Antibodies GTX73019 (GeneTex, Irvine, CA), and Santa Cruz antibodies C-20, H81, and D-6 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) all had very similar staining patterns, we chose to use the C-20 antibody, because a blocking peptide was available to confirm specificity. The rabbit anti-VDR antibody C-20 was raised against a region within the last 50 amino acids on the C-terminal end of the rat VDR protein. The antibody recognized a band of the expected size (50–55kDa) as well as several bands that have previously been shown with this antibody and other VDR antibodies (Gonzalez *et al*., 2008; Nangia *et al*., 1998). The sheep anti-CYP27B1 (The Binding Site, Birmingham, UK) was raised against a murine CYP27B1 peptide (RHVELREGEAAMRNQGKPEEDMPS) and recognized two bands, corresponding to sizes expected for full length CYP27B1 (56kDa) and a known splice variant that has no enzymatic activity (25kDa) (Diesel *et al.*, 2005). The goat anti-CYP24 G-15 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, Ca) was raised against 15 amino acids between amino acids 400–450 of the human CYP24a protein and recognized a single band corresponding to the expected size (55kDa). All western blot bands and immunofluorescent staining with the above antibodies was almost completely abolished after overnight preincubation of the primary antibodies with the following blocking peptides (1:5 antibody:blocking peptide): VDR C-20 (Santa Cruz Biotechnology Inc., Santa Cruz, Ca), CYP27B1 G-20 (Santa Cruz Biotechnology Inc., Santa Cruz, Ca), CYP27B1 G-15 (Santa Cruz Biotechnology Inc., Santa Cruz, Ca).

Peripherin selectively identifies unmyelinated neurons (Goldstein *et al.*, 1991). Because of potential interactions with double staining, two different peripherin antibodies were used. Chicken IgY anti-peripherin AB9282 (Millipore, Billerica, MA) was raised against recombinant peripherin protein and rabbit anti-peripherin AB1530 (Millipore, Billerica, MA) was raised against a trp-E fusion protein containing all but the four N-terminal amino acids. The rabbit anti-peripherin antibody has been previously characterized (Tseng *et al.*, 2008), and the percentage of DRG neurons expressing peripherin was similar to other accounts (Table 1) (Goldstein *et al.*, 1991). Double staining of DRG with both the chicken and rabbit antibodies revealed identical staining patterns.

Calcitonin gene-related peptide (CGRP) resides within NGF-dependent sensory neurons (Lawson, 1992), and again two separate antibodies were used. Goat anti-CGRP P01256 (AbD Serotec, Raleigh, NC) was raised against synthetic rat CGRP conjugated to gamma globulin, and sheep anti-CGRP CA1137 (Enzo Life Sciences, Plymouth Meeting, PA) was raised against synthetic rat CGRP conjugated to bovine serum albumin. These two antibodies had identical staining patterns, which have been previously characterized

(Ruscheweyh *et al.*, 2007; Yasuhara *et al.*, 2008), and the percentage of DRG neurons expressing CGRP was similar to that reported by others (Table 1) (Lawson, 1992).

Western Blots

Pooled left DRGs were homogenized in 350μl of RP1 buffer (Machery-Nagel, Düren, Germany) with 3.5μl of β-mercaptoethanol (Sigma-Aldrich Corp, St. Louis, MO) and proteins extracted using a Protein/RNA Nucleospin kit (Machery-Nagel, Düren, Germany). Proteins were separated side by side on 4–12% Bis-Tris precast gels (Invitrogen, Carlsbad, Ca) and transferred to PVDF membrane (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat milk, 0.1% bovine serum albumin, and 2% normal serum from the secondary antibody host. Blots were probed overnight at 4°C using anti-VDR C-20 (1:50 rabbit IgG, Santa Cruz), anti-CYP27B1 (1:50 sheep IgG, The Binding Site), or anti-CYP24 G-15 (1:50 goat IgG, Santa Cruz). Secondary antibodies conjugated to alkaline phosphatase or horseradish peroxidase were added for 2hr at room temperature. Blots were developed with either Biorad Immuno-Star AP substrate (Bio-Rad, Hercules, CA) or Thermo Scientific SuperSignal Chemiluminescent substrate (Rockford, IL). Blots were stripped (Restore Western Blot Stripping Buffer, Thermo Scientific, Rockford, IL) and reprobed with 1:2000 mouse anti-GAPDH (Millipore, Billerica, MA). Images were acquired on a Molecular Imager ChemiDoc XRS system (Bio-Rad, Hercules, CA)and analyzed with Quantity One software (Bio-Rad, Hercules, CA). Adjusted volumes ($Intensity_{*}mm^2$) of bands of interest were normalized to GAPDH in the same lane, and values for animal replicates averaged. Significance ($p \leq 0.05$) was determined by student's t-test. The images presented here have been cropped to show representative lanes/bands and adjusted for brightness and contrast.

Immunofluorescence

DRGs from each animal were sectioned at a thickness of 10μm and stored at −80°C. Thawed sections were post-fixed 30 min in 4% formaldehyde freshly-prepared from paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in phosphate buffered saline (PBS). Slides were incubated 20 min in blocker containing 30% normal serum (Equitech-Bio Inc., Kerrville, TX) from the secondary antibody host, 0.5% triton X-100 (Sigma-Aldrich, St. Louis, MO), 0.54% ammonium chloride (Sigma-Aldrich, St. Louis, MO), and 0.07% porcine gelatin (Sigma-Aldrich, St. Louis, MO). Sections were incubated overnight at room temperature with primary antibody at the following dilutions: 1:50 rabbit anti-VDR C-20, 1:25 Sheep anti-CYP27B1, 1:25 goat anti-CYP24 G-15, 1:1000 chicken IgY (or 1:400 rabbit anti-peripherin, 1:200 goat or Sheep (Biomol) anti-CGRP. Isolectin IB4 (IB4) conjugated to Alexa Flour 488 (Invitrogen, Carlsbad, Ca), which identifies unmyelinated sensory neurons that are responsive to the GDNF family of ligands (Ambalavanar and Morris, 1993; Bennett *et al.*, 1998), was added to slides for 20min between the primary and secondary antibodies at a concentration of 2μg/ml in PBS containing 1mM $CaCl_2$ (Fisher Scientific, Pittsburgh, PA). Nuclei were stained with 400 nM 4′,6-diamidino-2-phenylindole (DAPI, Invitrogen, Carlsbad, CA) for 10 min before the addition of secondary antibodies. Secondary antibody incubations were conducted at room temperature for 2 hrs at the following concentrations: 1:200 goat anti-chicken IgY Cy2, 1:500 donkey anti-rabbit Alex Flour 647 (Invitrogen, Carlsbad, CA), 1:200 donkey anti-sheep Cy3 (Jackson ImmunoResearch, West Grove, PA), 1:1000 donkey anti-sheep Alexa Fluor 488 (Invitrogen, Carlsbad, CA), or 1:200 donkey anti-goat Cy3 (Jackson ImmunoResearch, West Grove, PA). All antibodies were diluted in PBS containing 23% donkey serum, and slides were rinsed between staining steps. No staining was observed when primary antibodies were omitted and staining was almost completely abolished after preincubation of the primary antibodies with their blocking peptides.

Neuronal Quantitation

We quantitatively assessed immunoreactivity (ir) of vitamin D–related proteins and neural markers in the L5 DRG, because its relatively large size provides a substantial number of neurons to sample. Moreover, L5 DRG should have very few, if any, projections to the ovary, reproductive tract, or incision sites and therefore should not be damaged by the ovariectomy procedure (Berkley *et al.*, 1993; Steinman *et al.*, 1992; Takahashi *et al.*, 2003; Tanaka *et al.*, 2002). (No gross differences were observed between staining in L5 and the other vertebral levels of DRG for any of the proteins examined.) For each stain, sections 250μm apart throughout each ganglion were imaged on a Nikon 80i fluorescent microscope. A standard threshold value was determined for each stain, which appeared to differentiate real staining from background fluorescence. This threshold value was used to create a mask for each image so that neurons with clear DAPI-stained nuclei and staining intensities above the standard threshold could be manually counted without bias using Metamorph software. Counting sections 250μm apart throughout the ganglion (3–5 sections) yielded an average of 428±42 total neurons with clearly stained nuclei for each DRG. We further assessed cell types in which vitamin D-associated proteins reside by co-immunostaining for peripherin, CGRP, or IB4 and using the same color thresholding techniques for merged images as described above. We also characterized the intracellular localization of VDR to determine if; 1) Intensely stained VDR-immunoreactive aggregates were present within the DAPI-stained nuclear area, or 2) VDR-immunoreactive-staining above the standard threshold was found outside the DAPI-stained nuclear area for each neuron. For each animal and stain, percentages of labeled neurons were computed from averages of all sections analyzed, and data are presented as the mean ± standard error. Significance ($P \leq 0.05$) was determined by ANOVA followed by post-hoc analysis using Student-Newman-Keuls (SNK) or student's t-test. For publication images were pseudocolorized and adjusted for brightness and contrast.

Results

Vitamin D-related proteins in dorsal root ganglia of rats at estrus Vitamin D receptor

We assessed DRGs from intact female rats in estrus for the presence of VDRs. Western blots of total DRG protein revealed a strong band at approximately 60kDa. Two fainter bands were also consistently observed at 41kDa, and 24kDa and three very faint bands at 74kDa, 65kDa, and 50kDa could also be identified (Fig. 1). Immunofluorescence of sectioned DRG revealed VDR-ir in neurons, but no VDR-ir surrounding the neurons or within central nerve bundles where satellite glia and Schwann cells are located (Fig. 2A). Most neurons displayed VDR-ir localized to small nuclear aggregates; however, some neurons also had diffuse staining throughout the cell body or patchy membrane-localized staining. For this study we will refer to diffuse VDR-ir as cytoplasmic, although some of this staining may be confined to vesicles dispersed throughout the cytoplasm.

The percentage of total neurons with nuclear (nVDR) or cytoplasmic (cVDR) VDR-ir was quantified in L5 DRG (Fig. 2G). About 71% of all neurons express nVDR, while 10% had cVDR staining and 8% had both nVDR and cVDR-ir.

To determine whether VDR is differentially expressed by small or large fiber neurons, DRG sections were co-immunostained for VDR and the small fiber specific intermediate filament peripherin (Fig. 3A). Peripherin-positive and peripherin-negative neurons with clear DAPI stained nuclei were counted (DAPI-staining is not shown). In intact rats, the percentage of neurons with nVDR did not differ between the peripherin-negative and peripherin-positive neuronal subpopulations (Fig. 3G). On the other hand, cVDR was preferentially expressed by small fiber peripherin-positive neurons (Fig. 3H). The percentage of peripherin-ir

neurons expressing cVDR was more than twice that of peripherin-negative neurons (p=0.002).

To determine whether VDR is differentially expressed by a specific subset of small fiber neurons, DRG sections were co-labeled for VDR and IB4 (Fig. 3B), which stains small fiber neurons responsive to the GDNF family of ligands, or CGRP (Fig. 3C), which is found in NGF-dependent neurons. nVDR expression did not differ between the two neuronal sub-types (Fig. 3I), while the percentage of CGRP neurons that expressed cVDR was almost three times that of IB4-positive neurons (Fig. 3J; p=0.016).

CYP27B1

We assessed whether DRG from intact female rats in estrus express CYP27B1, the enzyme that converts circulating 25(OH)D3 into active 1,25$(OH)_2$D3. Western blots revealed two bands that migrated at approximately 56kDa and 25kDa (Fig. 1).

Immunofluorescent-staining of sectioned DRG revealed CYP27B1-ir in DRG neurons but not glia (Fig. 2B). CYP27B1-ir was diffuse throughout neural cell bodies. The percentage of total neurons expressing CYP27B1 was about 22% (Fig. 2H).

DAPI-stained DRG sections were immunofluorescently-labeled for CYP27B1 and peripherin to determine whether CYP27B1 is differentially expressed by small or large fiber neurons (Fig. 4A, DAPI-staining is not shown). The percentage of peripherin-ir neurons expressing CYP27B1 was more than three times that of peripherin-negative neurons (Fig 4G; p<0.001).

DRG sections were immunofluorescently-labeled for CYP27B1 and IB4 (Fig. 4B) or CGRP (Fig. 4C) to determine whether expression occurs in specific neuronal subsets. The percentage of CGRP-ir neurons that expressed CYP27B1 was more than two times the percentage of IB4-positive expressing CYP27B1 (Fig. 4H; p=0.003).

CYP24

Western Blot was used to determine whether DRG from intact female rats in estrus express CYP24, an enzyme involved in the degradation of 1,25$(OH)_2$D3 and the production of other vitamin D metabolites. A single faint band of the expected size of 55kDa was identified on western blots run with total DRG protein (Fig. 1).

Immunofluorescence of sectioned DRG revealed CYP24-ir in DRG neurons but not glia (Fig. 2C). Like CYP27B1-ir, CYP24-ir was diffuse throughout neural cell bodies. The percentage of total neurons with CYP24 was quantified in the L5 DRG of intact female rats in estrus. About 17% of all neurons express CYP24 (Fig. 2I).

To determine whether CYP24 is differentially expressed by large or small fiber neuronal cell types, DRG sections were co-labeled for CYP24 and peripherin (Fig. 5A). CYP24 was preferentially expressed by small fiber peripherin-positive neurons (Fig. 5G). The percentage of peripherin-ir neurons expressing CYP24 was one and a half times that of peripherin-negative neurons.

We assessed the extent to which CYP24 is expressed by peptidergic or non-peptidergic small fiber neurons. DRG sections were immunofluorescently-labeled for CYP24 and CGRP (Fig. 5C) or IB4 (Fig. 5B). The percentage of CGRP-positive neurons expressing CYP24 was about four times more than the percentage of IB4-positive neurons expressing CYP24 (Fig. 5H; p<0.001).

cVDR colocalizes with CYP27B1 and CYP24

Because cVDR, CYP27B1, and CYP24 were all preferentially expressed by peripherin-ir, CGRP-ir neurons, we co-stained DRG for cVDR and CYP27B1 (Fig. 6A) or CYP24 (Fig. 6B) to determine if they are expressed within the same neurons. Most neurons with cVDR-ir were observed to have CYP27B-ir and CYP24-ir, showing that neurons with cVDR typically have proteins necessary to locally regulate $1,25(OH)_2D3$ concentrations.

Effect of ovariectomy (OVX) on Vitamin D-related proteins in dorsal root ganglia VDR

Having verified that DRG neurons from intact female rats express VDR, CYP27B1, and CYP24, we assessed whether expression of these proteins is affected by OVX. We began by examining whether OVX alters VDR protein levels in total DRG lysates (Fig. 7A). The only VDR isoform to change significantly following OVX was the 60kDa band, which was reduced by 51% compared to lysates from intact rats in estrus ($p=0.037$).

Immunohistochemistry revealed that, as in intact rats, VDR-ir in ovariectomized rats was distributed in nuclear and cytoplasmic compartments in neurons, but not glia (Fig. 2D). Approximately 66% of neurons in DRG from ovariectomized rats express nVDR, 8% express cVDR, and 6% express both (Fig. 2G); only the modest reduction in nVDR was significant ($p=0.038$).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for VDR and peripherin (Fig. 3D). The percentage of peripherin-ir and peripherin-negative DRG neurons expressing nVDR in ovariectomized rats did not change (Fig. 3G). However, the percentage of peripherin-ir neurons with cVDR was reduced, so that there was no longer any difference in the percentage of peripherin-ir and peripherin-negative neurons expressing cVDR in ovariectomized rats (Fig. 3H).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for VDR and IB4 (Fig. 3E) or CGRP (Fig. 3F). The percentage of DRG neurons expressing nVDR in ovariectomized rats was comparable in CGRP-ir and IB4-positive neurons (Fig. 3I). However, there was a 63% decrease in the percentage of CGRP-ir expressing cVDR following OVX (Fig. 3J; $p=0.009$).

CYP27B1

Western blots of total DRG protein showed that while there was no change in expression of the 25kDa CYP27B1 band, expression of the 56kDa CYP27B1 band dropped by 31% following OVX (Fig. 7B; $p=0.042$).

CYP27B1-ir in DRG from ovariectomized rats was found in neurons, but not glia (Fig. 2E). The average percentage of total neurons expressing CYP27B1 dropped from 22±4% to 10±2% following OVX, but this change did not achieve statistical significance (Fig. 2H; $p=0.063$).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for peripherin and CYP27B1 (Fig. 4D). Ovariectomy did not affect the small proportion of peripherin-negative neurons expressing this enzyme. (Fig. 4G). However, OVX resulted in a 57% decrease in proportion of peripherin-ir neurons expressing CYP27B1 ($p=0.003$) to a level comparable to that of peripherin-negative neurons (Fig. 4G).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for CYP27B1 and IB4 (Fig. 4E) or CGRP (Fig. 4F). Ovariectomy did not significantly alter the proportion of IB4-positive neurons expressing CYP27B1 (Fig. 4H). In contrast, there was a 61% drop in the proportion of CGRP-ir DRG neurons with CYP27B1-ir after OVX (Fig. 4H; $p<0.001$),

such that percentages of neurons expressing this enzyme were comparable in IB4 and CGRP-ir subpopulations.

CYP24

Western blots of total DRG protein showed that there was no significant change in total CYP24 levels following OVX (Fig. 7C). As in rats at estrus, CYP24-ir was present exclusively in neurons (Fig. 2F), and immunostaining showed no change in the percentage of total neurons expressing CYP24 following OVX (Fig. 2I).

DAPI-stained DRG sections from ovariectomized rats were co-labeled for peripherin and CYP24 (Fig. 5D). As with rats in estrus, CYP24-ir was preferentially expressed by small fiber peripherin-positive neurons in OVX rats (Fig. 5G). Ovariectomy did not alter the proportion of peripherin-ir or peripherin negative neurons displaying CYP24-ir (Fig. 5G).

DAPI-stained DRG sections from OVX rats were co-labeled for CYP24 and IB4 (Fig. 5E) or CGRP (Fig. 5F). As in estrus, CYP24-ir was present predominantly within CGRP-ir neurons, with only small numbers of IB4-positive neurons expressing this enzyme (Fig. 5H). Ovariectomy did not alter numbers of either CGRP-ir or IB4-positive neurons expressing CYP24 (Fig. 5H).

Changes in neural markers following ovariectomy

We assessed whether changes in neuronal protein expression following OVX could contribute to observed differences in expression of vitamin D-related proteins. The percentage of total neurons expressing peripherin was 63% in intact rats and underwent a small but significant decline to 59% following OVX ($p=0.042$, Table 1). Within the peripherin-positive population of DRG neurons, OVX resulted in a 38% decline in the percentage of neurons expressing CGRP-ir, while the IB4-positive population was unaffected (Table 1).

The small decrease in peripherin-positive neurons resulted in a proportional increase in peripherin-negative neurons, but the small numbers of these neurons expressing CGRP or IB4 were unaffected (Table 1). The percentages of IB4-positive and CGRP-ir neurons that expressed peripherin-ir were unaltered by OVX (Tables 1).

Discussion

The central finding of this study is that adult rat sensory neurons express proteins associated with vitamin D signaling pathways. Nuclear VDR is widely distributed within all types of DRG neurons suggesting broad transcriptional actions of this hormone. Membrane and cytoplasmic VDR, perhaps indicative of rapid signaling pathways, is present selectively in unmyelinated, CGRP-ir neurons. This same subpopulation contains vitamin D metabolizing enzymes, implying close regulation of vitamin D metabolite levels. Ovarian hormones may play an important role in regulating neuronal responsiveness to $1,25(OH)_2D_3$ by up-regulating both its activating enzyme and its cytoplasmic receptor. Because vitamin D deficiency is strongly associated with musculoskeletal pain and this is exacerbated by the loss of ovarian hormones, it is important to consider the role of these convergent systems in regulating nociceptor function.

Vitamin D signaling in the central nervous system

Vitamin D is becoming recognized as an important hormone in nervous system health. VDR is expressed by neurons, oligodendrocytes, and astrocytes within the limbic system, basal ganglia, spinal cord, cerebellum, and cerebral cortex (Baas *et al.*, 2000; Eyles *et al.*, 2005;

Glaser *et al.*, 1999; Neveu *et al.*, 1994; Perez-Fernandez *et al.*, 1997; Prufer *et al.*, 1999; Walbert *et al.*, 2001). VDR localization within both neuronal nucleus and cytoplasm has been described (Glaser *et al.*, 1999; Prufer *et al.*, 1999; Racz and Barsony, 1999). Studies have also shown that neurons and glia in brain express CYP27B1 (Eyles *et al.*, 2005; Zehnder *et al.*, 2001), and CYP24 mRNA has been detected in glioma and primary glial cell culture (Naveilhan *et al.*, 1993), suggesting that the brain can regulate local $1,25(OH)_2D_3$ concentrations. Vitamin D signaling has been linked to cell survival, proliferation, and differentiation, and is especially important during brain development (Levenson and Figueiroa, 2008). In fact, gestational vitamin D deficiency is thought to be associated with long term cognitive disorders such as anxiety and schizophrenia (Levenson and Figueiroa, 2008). In the adult, vitamin D supplementation has been proposed to improve outcomes in multiple sclerosis, Parkinson's disease, and traumatic brain injury (Cekic *et al.*, 2009; Myhr, 2009; Newmark and Newmark, 2007). Thus, accumulating evidence suggests that vitamin D signaling plays important roles in the central nervous system.

Vitamin D-related proteins in adult rat DRG

Much less is known regarding the role of vitamin D in the peripheral nervous system. VDR has been identified in fetal (E12-E21) DRG neurons but not glia (Johnson *et al.*, 1996; Veenstra *et al.*, 1998) (although VDR mRNA was reported in cultured sciatic nerve Schwann cells (Cornet *et al.*, 1998)). However, the presence of VDRs and related proteins in the adult peripheral nervous system is not well documented.

Our western blot findings show that VDR protein is present within adult rat DRG, and immunocytochemical analyses show that it is found in most neurons. VDR can regulate transcription by forming homodimers or heterodimers with 9-cis retinoid X receptor (RXR) which then translocate to the nucleus (Shaffer and Gewirth, 2004). Consistent with a transcriptional role, nuclear VDR was observed in the majority of DRG neurons as small discrete foci (Glaser *et al.*, 1999; Prufer *et al.*, 1999); because these may be absent from a given section plane of a given neuronal nucleus, we may have underestimated their actual prevalence. These foci are believed to represent binding of ligand-activated RXR/VDR heterodimers to DNA, thus corresponding to sites of transcription (Prufer *et al.*, 2000). Because nuclear VDR was distributed widely throughout all DRG neural subtypes, VDR is likely to act as a classical transcription factor in most adult DRG neurons.

We also observed a subset of neurons where VDR-ir was localized to the cytoplasm or plasma membrane. While some of the cytosolic VDR could represent *de novo* synthesis or ligand-deficient VDR dimers (Prufer *et al.*, 2000; Racz and Barsony, 1999), another explanation is that this VDR fraction is involved in rapid signaling. The most prominent band disclosed in DRG homogenates had a mass of 60kDa, and this isoform reportedly is exclusive to membrane and cytosolic subcellular fractions (Gonzalez Pardo *et al.*, 2008). In cardiomyocytes, cVDR serves as a reserve which, upon $1,25(OH)_2D_3$ binding, translocates to cardiac t-tubules (Tishkoff *et al.*, 2008). The patchy VDR-ir pattern localized to the plasma membrane of some DRG neurons suggests a distribution to distinct membrane microdomains, and is consistent with a role for VDR in rapid signaling in sensory neurons (Huhtakangas *et al.*, 2004). For example, $1,25(OH)_2D_3$ can influence calcium channel activity, leading to rapid increases in intracellular calcium concentration and production of second messengers (Losel *et al.*, 2003). Thus, in addition to its transcriptional role within all DRG neuronal populations, VDR may exert rapid signaling in a selected subpopulation of neurons.

VDR signaling in DRG neuronal subpopulations

Selective neuronal markers provide insight as to how VDR signaling may vary as a function of neuronal subtype. Neurons lacking the intermediate filament protein peripherin are predominantly large myelinated neurons (Goldstein *et al.*, 1991), which are involved in tactile sensation and proprioception (Casellini and Vinik, 2007; Muller *et al.*, 2008; Nardone *et al.*, 2007). These neurons contained nVDR but were mostly devoid of both CYP27B1-ir and CYP24-ir. This implies that large sensory neurons may be influenced by VDR transcriptional signaling, but that VDR activation is determined passively by levels of circulating $1,25(OH)_2D_3$, as they likely lack the ability to regulate local $1,25(OH)_2D_3$ levels. The functional significance of this signaling pathway is unclear, but it is noteworthy that vitamin D deficiency in humans is associated with increased incidence in falls (Bischoff-Ferrari *et al.*, 2004; Faulkner *et al.*, 2006), which may suggest altered proprioceptive function of large sensory fibers.

Neurons containing peripherin-ir represent unmyelinated C-fibers, which are associated with chemoreception, thermal sensation, and pain, burning and itching. As with large myelinated neurons, the majority of these also showed VDR-ir localized to the nucleus. However, these peripherin-ir neurons also include the subgroup in which VDR is localized to the cytoplasm, most often in conjunction with nuclear staining. It is well known that C-fiber neurons can be further characterized based on their neurotrophin dependencies and peptide content, and our colocalization studies show that the cytoplasmic form of VDR is distributed selectively in the NGF-dependent, CGRP-ir neurons, with approximately one third displaying cVDR; these neurons conduct information regarding pain and temperature, and antidromically release neuropeptides that promote vasodilation and extravasation, thus contributing to inflammation (Birklein and Schmelz, 2008). These findings suggest that vitamin D can activate multiple signaling pathways, which may functionally influence a subpopulation of neurons with pain-sensing capabilities.

Our observations regarding VDR distribution in the DRG suggest that some neurons may be more strongly influenced by vitamin D than others. If so, then local vitamin D metabolite levels for those neurons may require closer regulation. Findings concerning vitamin D-related proteins provide evidence that this is the case. Hence, the prohormone converting enzyme CYP27B1 is enriched specifically in peripherin-ir, CGRP-ir neurons, as is CYP24, which could be involved in the inactivation of $1,25(OH)_2D_3$ or perhaps the formation of other metabolites such as $24,25(OH)_2D_3$. Further, these enzymes co-localize selectively within neurons displaying cytoplasmic VDR. Collectively, these findings are consistent with the hypothesis that DRG peptidergic neurons possess vitamin D signaling pathways, whose gain can be modulated by altering levels of cVDR, or by regulating levels of vitamin D metabolic enzymes.

Regulation of VDR and CYP27B1by ovarian hormones

One likely factor in regulating vitamin D-related proteins in DRG is female reproductive hormone status. Ovarian hormones have been found to promote the formation of $1,25(OH)_2D_3$ by enhancing CYP27B1 expression and by inhibiting the expression of CYP24 (Lechner *et al.*, 2006; Tanaka *et al.*, 1978). They are also reported to promote the expression of VDR (Gilad *et al.*, 2005). In cancer models there has been some progress in uncovering how estradiol regulates VDR expression. Estradiol binds membrane bound ERβ receptors, which signal through ERK1/2 to increase transcripton of VDR (Gilad et al., 2005). Research from another group has shown that estrogen may regulate VDR promoter activity of exon 1C through SP1 sites, which can be mediated through ERα or ERβ (Wietzke et al., 2005). To determine whether ovarian hormones regulate VDR or enzyme expression in DRG neurons, we compared vitamin D-related protein expression in DRG from cycling

rats at estrus to that of ovariectomized rats. Estrus occurs several hours after the natural surge in reproductive hormones including estradiol, progesterone, and prolactin (Freeman, 1994), when hormone-responsive changes in protein expression are most apt to be evident. In contrast, rats following OVX have sustained depression of ovarian hormones similar to that which occurs naturally in menopause, albeit occurring at a younger age and more abruptly than would occur normally.

Consistent with reported findings for other tissues, OVX resulted in decreased expression of VDR and CYP27B1. In protein blots, the cytoplasm/membrane-specific 60kDa isoform of VDR was reduced by 51%, and the 56kDa full length CYP27B1 band was reduced by 31%. Immunofluorescence examination showed that the numbers of neurons with cVDR and CYP27B1 expression were both diminished, and this decrease occurred exclusively within the peripherin-ir, CGRP-ir population. In contrast, no significant changes occurred in nVDR or CYP24. Therefore, based on these findings in OVX rats, a decline in ovarian hormones would not only down-regulate cVDR expression, but would also decrease the amount of CYP27B1 available to convert prohormone to active $1,25(OH)_2D_3$. At the same time, levels of the CYP24 remain unchanged, thereby favoring a reduction in levels of $1,25(OH)_2D_3$. Accordingly, low ovarian hormone levels may impair vitamin D signaling in peptidergic nociceptor neurons by diminishing levels of both cyotplasmic receptor and ligand.

While these observations are consistent with a reduction in the numbers of neurons in which cVDR signaling occurs, a caveat is that changes in reproductive hormones could influence marker expression, thereby introducing error to our counts of neuronal subpopulations containing vitamin D-related proteins. While hormone-related changes in IB4-positive neurons have not been reported (and were not detected here), it is known that estrogens increase CGRP expression (Gangula *et al.*, 2000; Mowa *et al.*, 2003). Consistent with these reports, we found that OVX resulted in a reduction of roughly 1/3 in the number of neurons expressing CGRP-ir. Of those retaining the CGRP phenotype, cVDR and activating enzyme expression were reduced by over 60%. This occurred without concomitant increases in cVDR or CYP27B1 in other cell phenotypes that might imply changes due to altered marker expression. It therefore appears that ovarian hormone deficiency selectively affects a subpopulation of DRG neurons, where both peptide phenotype and VDR signaling are dramatically reduced.

Vitamin D, ovarian hormones, and pain

While an association between pain and vitamin D deficiency has been observed for at least 20 years (Masood *et al.*, 1989), this is often attributed to bone and muscle pathologies. Muscle symptoms, including pain, precede the appearance of biochemical indicators of bone pathology (Glerup *et al.*, 2000; Masood *et al.*, 1989), suggesting that bone is not the only source of musculoskeletal pain. Vitamin D deficiency does lead to atrophy of type II muscle fibers (Glerup *et al.*, 2000); however, type II muscle fiber atrophy is non-specific and does not in itself cause pain (Mastaglia and Hilton-Jones, 2007). Based on our findings, it seems likely that vitamin D may act directly on sensory nerves to modulate pain. Moreover, the extent to which vitamin D deficiency produces musculoskeletal pain appears to be strongly linked to estrogen status. For example, musculoskeletal pain occurs commonly in hypoestrogenemia resulting from naturally occurring menopause (Andersson *et al.*, 1993; Bergman *et al.*, 2001; Croft *et al.*, 1993; Dugan *et al.*, 2006; Greendale *et al.*, 1998; White *et al.*, 1999; Wolfe *et al.*, 1995) or pharmacotherapy using aromatase inhibitors or gonadotropin-releasing hormone agonists (Felson and Cummings, 2005; Friedman *et al.*, 1993; Garreau *et al.*, 2006; Morales *et al.*, 2006; Mouridsen, 2006). Musculoskeletal pain associated with menopause is ameliorated by hormone replacement therapy (Dugan *et al.*, 2006), implying a direct link between pain and hormone levels. It is not clear why musculoskeletal pain occurs with hypoestrogenemia. However, low estrogen levels

following aromatase inhibition are accompanied by low serum 25(OH)D3 levels, and high dose vitamin D_3 supplementation partially reverses the discomfort (Khan *et al.*, 2009; Waltman *et al.*, 2009), consistent with a link among estrogens, vitamin D and pain. However, it should be noted that high dose vitamin D_3 treatment does not fully alleviate pain symptoms. This may not be surprising given our findings that low ovarian hormone levels are also associated with diminished DRG neuronal expression of VDR and CYP27B1. Thus, hypoestrogenemia might hinder conversion of the therapeutic supplement to the active hormone and limit receptor-mediated signaling, thereby rendering dietary supplementation less effective. Collectively, these studies and the present findings underscore a need for additional research clarifying the relationships among vitamin D, steroid hormones, and sensory pathways.

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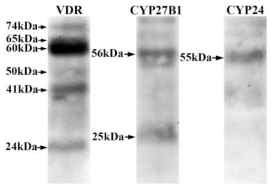

Figure 1.
VDR, CYP27B1, and CYP24 proteins are present in adult DRG. Western blots of extracted DRG proteins from intact rats in estrus were probed for VDR, CYP27B1, or CYP24. Samples were run alongside molecular markers in order to estimate protein size. Images are cropped to show representative lanes.

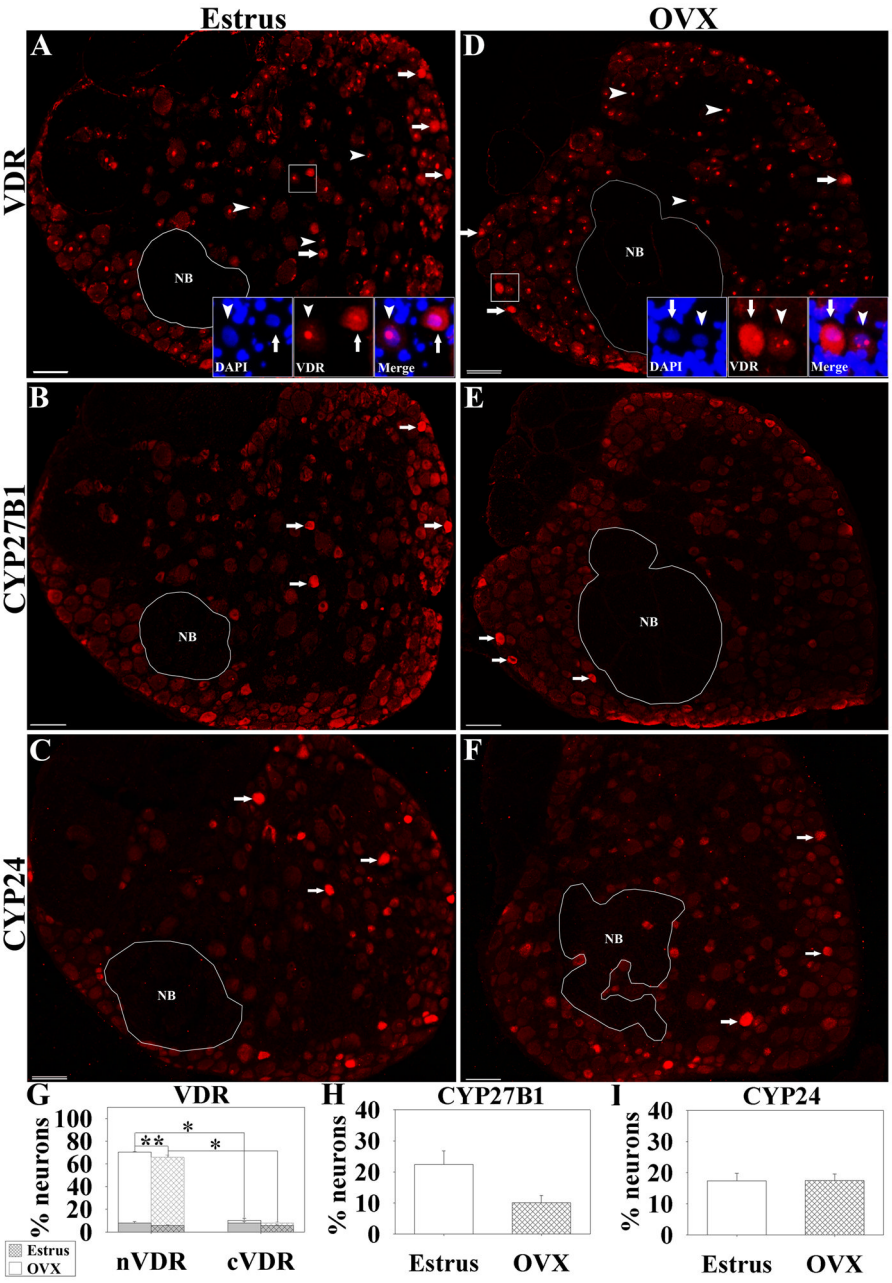

Figure 2.

Cellular localization of VDR, CYP27B1, and CYP24 in adult DRG. Cryosections of L5 DRG from intact rats in estrus (A–C) or ovariectomized (OVX) rats (D–F) were immunofluorescently labeled for VDR (A&D; red), CYP27B1 (B&E; red), or CYP24 (C&F; red), and nuclei were stained with DAPI (shown only in the insets of A and D; blue). Examples of neurons with nuclear-localized VDR are indicated in A and D by arrow heads, and neurons with intense cytoplasmic VDR staining are indicated by arrows. The insets show magnified examples of neurons with nuclear or nuclear and cytoplasmic VDR immunoreactivity. In B& E, examples of neurons with intense labeling for CYP27B1 are indicated by arrows. In C & F, neurons with intense labeling for CYP24 are indicated by arrows. In A–F staining is not consistent with glial expression of VDR, CYP27B1, or

CYP24 (ie. No staining surrounding neurons in satellite glia or in the Schwann cells of outlined nerve bundles (NB)). In G, the total proportion of neurons expressing VDR in estrus or after OVX is presented. The shaded area within each bar represents the proportion of neurons with both cytoplasmic and nuclear VDR staining. Significant differences between individual groups are shown by bracketing, p= *<0.001 and **0.038. The overall difference between Estrus and OVX expressing VDR was also significant, p=0.028. H and I present quantitative analysis of the percentages of neurons immunostained for CYP27B1 and CYP24, respectively. Scale bar = 100μm (10μm in insets).

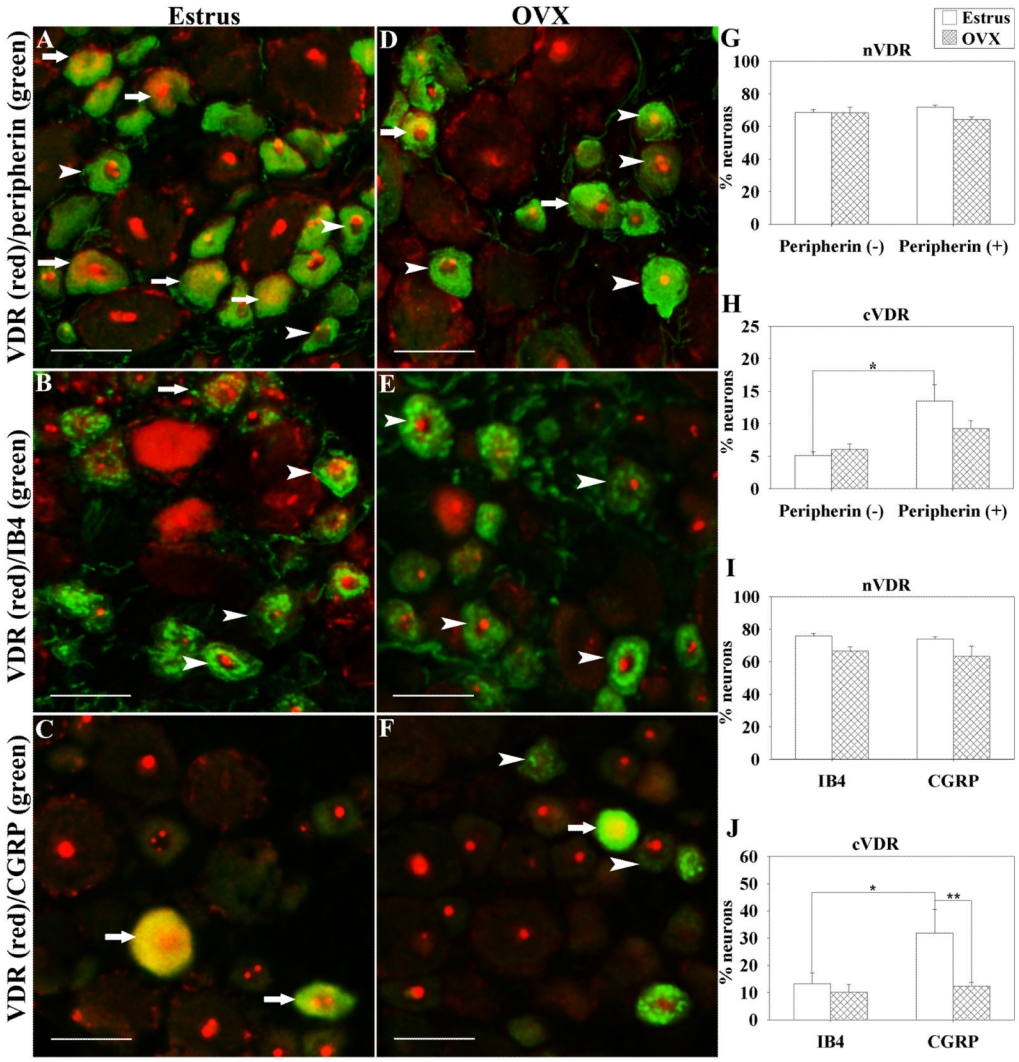

Figure 3.

Cellular colocalization of VDR with the neural markers peripherin, IB4, and CGRP. Cryosections of L5 DRG from intact rats in estrus (A–C) or ovariectomized rats (D–F) were immunofluorescently-labeled for VDR (A–F; red) and peripherin (A&D; green), IB4 (B&E; green), or CGRP (C&F; green), and the nuclei were stained with DAPI (A–F not shown). Neurons with nuclear VDR and neural markers are indicated by arrowheads, and neurons with cytoplasmic VDR and neural markers are indicated by arrows. G–J: The mean percentages of neurons with neural marker immunoreactivity that express nVDR or cVDR are presented in graphical form. Significant differences between individual groups are shown by bracketing, p= H) *0.002, J) *0.016 and **0.009. Overall differences between neural markers were significant for H) cVDR peripherin (+) vs. peripherin (−) (p=0.001) and J) cVDR IB4 vs. CGRP (p=0.039). Overall differences between Estrus and OVX were also significant J) p=0.027. Scale bar = 50μm.

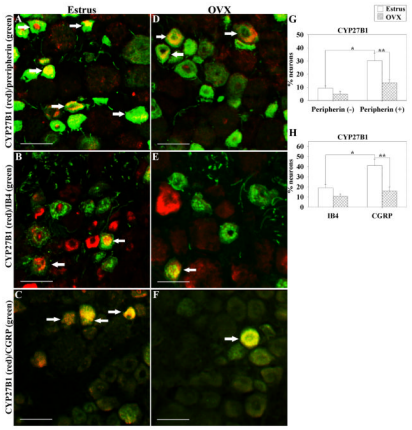

Figure 4.

Cellular colocalization of CYP27B1 with the neural markers peripherin, IB4, and CGRP. Cryosections of L5 DRG from intact rats in estrus (A–C) or ovariectomized rats (D–F) were immunostained for CYP27B1 (A–F; red) and peripherin (A&D; green), IB4 (B&E; green), or CGRP (C&F; green), and the nuclei were stained with DAPI (A–F not shown). Neurons with intense CYP27B1 and neural marker staining are indicated by arrows. G–J: The mean percentages of neurons with neural marker immunoreactivity that express CYP27B1 are illustrated in graphical form. Significant differences between individual groups are shown by bracketing, p= G) *<0.001 and **0.003, H) *0.003 and <0.001. Overall differences between neural markers was significant for G) CYP27B1 peripherin (+) vs. peripherin (−) (p<0.001) and J) CYP27B1 IB4 vs. CGRP (p=0.005). Overall differences between Estrus and OVX were also significant G) p=0.005 and J) p=0.001. Scale bar = 50μm.

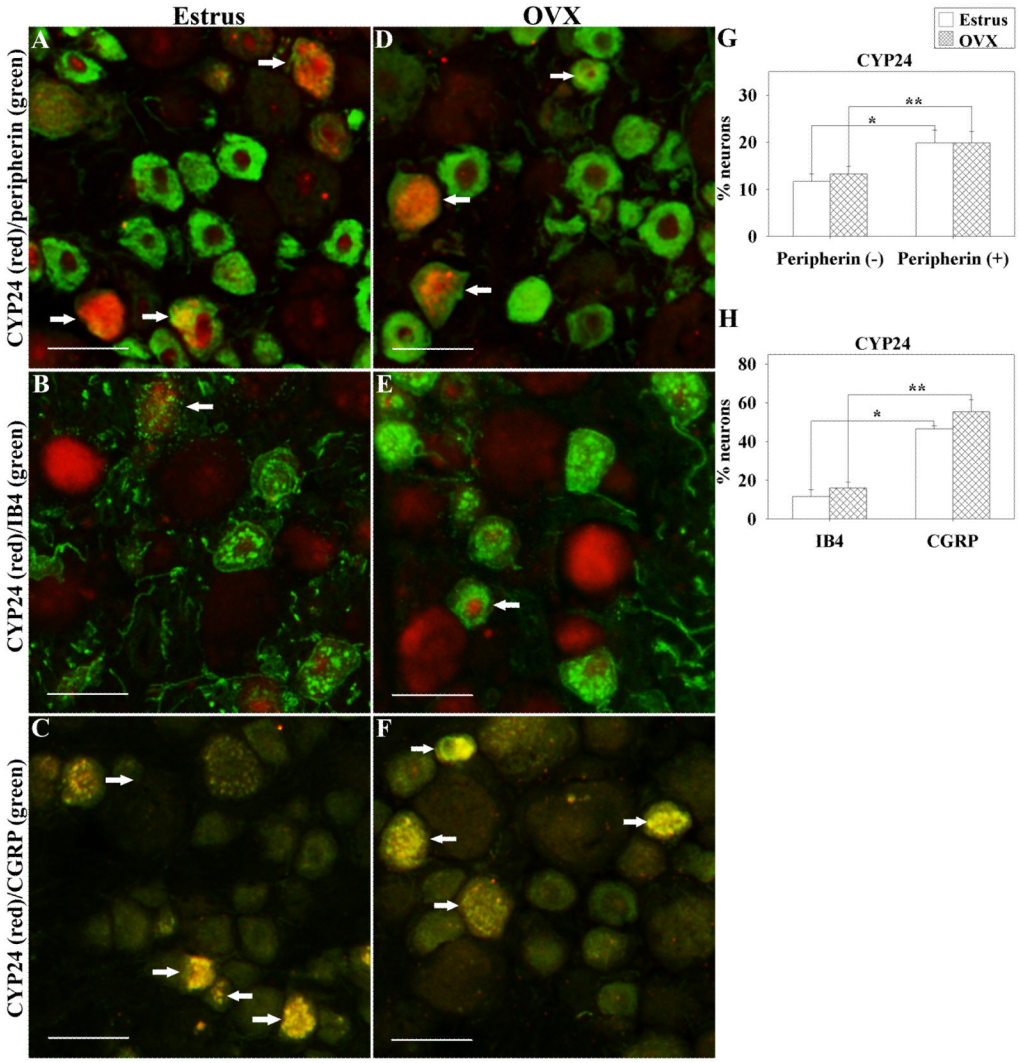

Figure 5.

Cellular colocalization of CYP24 with the neural markers peripherin, IB4, and CGRP. Cryosections of L5 DRG from intact rats in estrus (A–C) or ovariectomized rats (D–F) were immunostained for CYP24 (A–F; red) and peripherin (A&D; green), IB4 (B&E; green), or CGRP (C&F; green), and the nuclei were stained with DAPI (A–F not shown). Neurons with intense CYP24 and neural marker staining are indicated by arrows. G&H: The mean percentages of neurons with neural marker immunoreactivity that express CYP24 are illustrated in graphical form. Significant differences between individual groups are shown by bracketing, p= G) *0.024 and **0.005, J) *<0.001 and **0.001. Overall differences among neural markers were significant for G) CYP24 peripherin (+) vs. peripherin (−) (p=0.005) and J) CYP24 IB4 vs. CGRP (p<0.001). Scale bar = 50μm

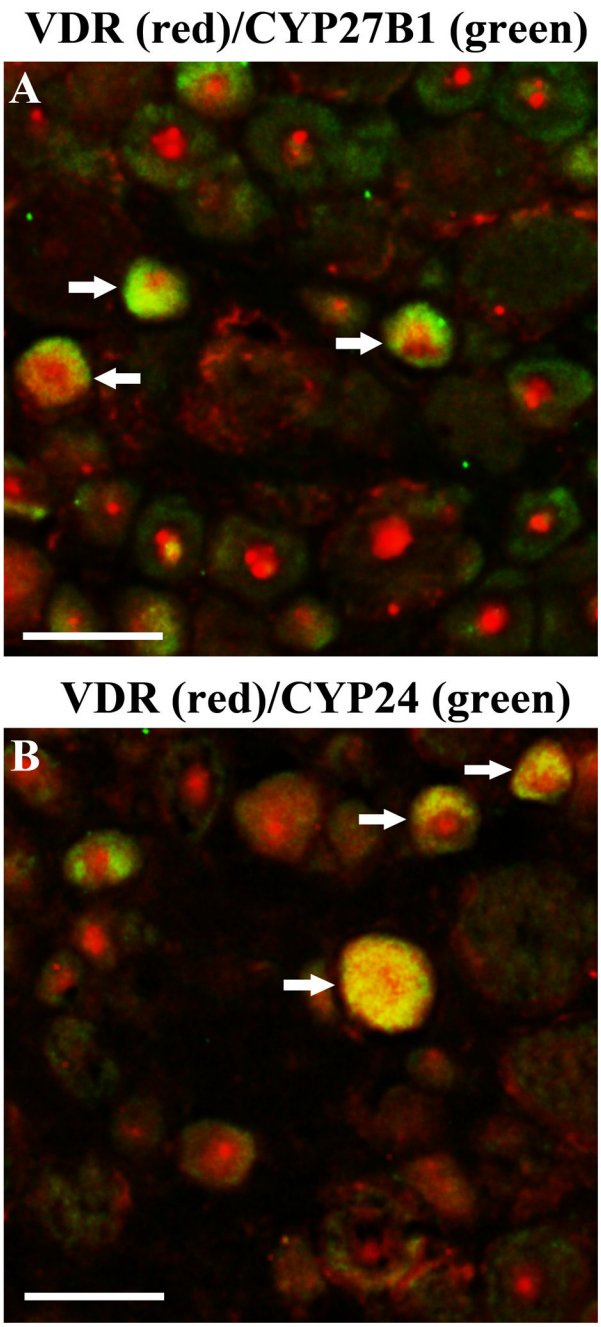

Figure 6.
Cellular colocalization of VDR with the enzymes CYP27B1 or CYP24. Cryosections of L5 DRG from rats in estrus were immunostained for VDR (red) and the enzymes CYP27B1 (A) or CYP24 (B) (green) and nuclei were stained with DAPI (not shown). Neurons co-labeled for each enzyme and cVDR are indicated by arrows. Scale bar = 50 μm.

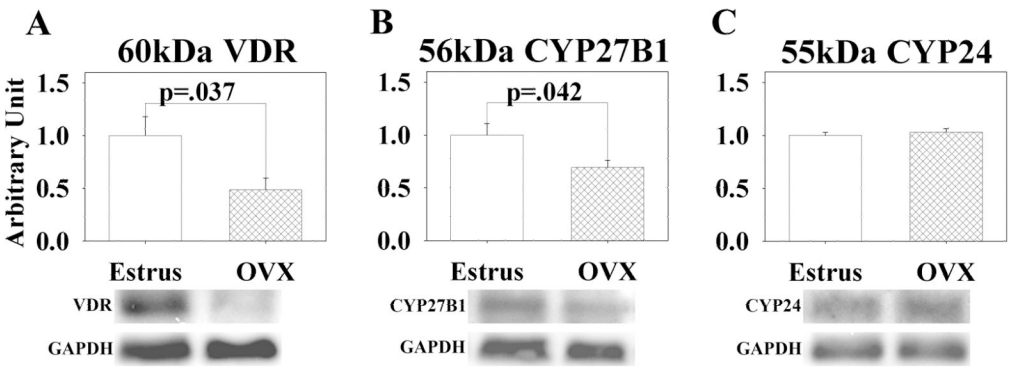

Figure 7.

DRG expression of VDR and CYP27B1, but not CYP24 is reduced following ovariectomy. Western blots of proteins extracted from DRG of intact rats in estrus or ovariectomized rats were probed for VDR (A), CYP27B1 (B), or CYP24 (C) and GAPDH (A–C) as a loading control. A) The 60kDa band of VDR showed a significant decrease in intensity following ovariectomy. B) The 56kDa band of CYP27B1 also showed a significant drop in intensity following ovariectomy. C) There was no significant change in CYP24 after ovariectomy. OVX n=5, Estrus n=4, 2–3 replicates per animal were averaged. Significance determined by student's t-test.

Table 1

Quantitative analysis of neuronal markers and effect of ovariectomy. This table shows the percentages of total neurons, peripherin-positive neurons, peripherin-negative neurons, CGRP-positive neurons, or IB4-positive neurons that express peripherin, CGRP, or IB4. % Estrus (% OVX). There was a significant drop in the percentage of total neurons expressing peripherin and peripherin-positive neurons co-expressing CGRP following ovariectomy, p=*0.042 and **0.049 by student's t-test.

	Total neurons %	Peripherin- positive neurons %	Peripherin- negative neurons %	IB4 neurons %	CGRP neurons %
Peripherin	*63±1 *(59±1)	100 (100)	0 (0)	90±2 (85±3)	85±2 (76±4)
IB4	35±2 (36±2)	54±4 (57±1)	9±2 (11±2)	100 (100)	Not determined
CGRP	43±7 (28±3)	**55±9 **(34±3)	18±2 (19±4)	Not determined	100 (100)