Development/Plasticity/Repair

Vitamin D Deficiency Promotes Skeletal Muscle Hypersensitivity and Sensory Hyperinnervation

Sarah E. Tague,[1,4] Gwenaëlle L. Clarke,[1,4] Michelle K. Winter,[2,4] Kenneth E. McCarson,[2,4] Douglas E. Wright,[3,4] and Peter G. Smith[1,4]

Departments of [1]Molecular and Integrative Physiology, [2]Pharmacology, Toxicology and Therapeutics, and [3]Anatomy and Cell Biology, and [4]Kansas Intellectual and Developmental Disabilities Research Center, University of Kansas Medical Center, Kansas City, Kansas 66160

Musculoskeletal pain affects nearly half of all adults, most of whom are vitamin D deficient. Previous findings demonstrated that putative nociceptors ("pain-sensing" nerves) express vitamin D receptors (VDRs), suggesting responsiveness to 1,25-dihydroxyvitamin D. In the present study, rats receiving vitamin D-deficient diets for 2–4 weeks showed mechanical deep muscle hypersensitivity, but not cutaneous hypersensitivity. Muscle hypersensitivity was accompanied by balance deficits and occurred before onset of overt muscle or bone pathology. Hypersensitivity was not due to hypocalcemia and was actually accelerated by increased dietary calcium. Morphometry of skeletal muscle innervation showed increased numbers of presumptive nociceptor axons (peripherin-positive axons containing calcitonin gene-related peptide), without changes in sympathetic or skeletal muscle motor innervation. Similarly, there was no change in epidermal innervation. In culture, sensory neurons displayed enriched VDR expression in growth cones, and sprouting was regulated by VDR-mediated rapid response signaling pathways, while sympathetic outgrowth was not affected by different concentrations of 1,25-dihydroxyvitamin D. These findings indicate that vitamin D deficiency can lead to selective alterations in target innervation, resulting in presumptive nociceptor hyperinnervation of skeletal muscle, which in turn is likely to contribute to muscular hypersensitivity and pain.

Introduction

Chronic musculoskeletal pain poses major social and economic burdens, given that approximately one-third of adolescents and one-half of adults suffer from this disorder (De Inocencio, 2004; Bergman, 2007). Up to 93% of those reporting nonspecific musculoskeletal pain are deficient in vitamin D (serum 25-hydroxyvitamin D[25(OH)D] <20 ng/ml [50 nmol/L]) (Plotnikoff and Quigley, 2003), suggesting that insufficient levels of this secosteroid may contribute to the etiology of musculoskeletal pain. There is evidence that dietary vitamin D supplementation can relieve musculoskeletal pain (Gloth et al., 1991; Glerup and Eriksen, 1999; de Torrenté de la Jara et al., 2006), leading some to promote vitamin D as a complementary or alternative medicine for nonspecific muscle pain. Nonetheless, the role of vitamin D in musculoskeletal pain remains controversial, as conditions that encourage vitamin D deficiency (obesity, sedentary lifestyle, senescence, female and menopausal) also predispose individuals to musculoskeletal pain (Bergman, 2007; Yetley, 2008). This study uses a rodent model to investigate whether vitamin D deficiency causes deep tissue mechanical hypersensitivity and explores potential mechanisms.

Vitamin D affects many cell types through nuclear vitamin D receptors that regulate gene expression and cell membrane VDRs that mediate nongenomic rapid responses. VDRs are activated by the vitamin D hormone metabolite, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$], which is converted from circulating 25(OH)D. We recently found that unmyelinated, peptidergic neurons in rat dorsal root sensory ganglia (DRGs) express nuclear, membrane, and cytoplasmic VDRs and the enzyme that converts 25(OH)D to ($1,25(OH)_2D$), suggesting that these presumptive pain-sensing neurons produce and respond to active vitamin D metabolites (Tague and Smith, 2011).

We hypothesized that vitamin D deficiency contributes to muscle hypersensitivity through direct effects on sensory nociceptor neurons. Reduced vitamin D dietary intake can induce persistent vitamin D deficiency in laboratory rats (Weishaar and Simpson, 1987), and we used this model to assess cutaneous and deep muscle sensitivity. However, reductions in serum calcium that can accompany vitamin D deficiency can affect many physiological functions (Demay, 2006), and can be corrected by administering a diet high in calcium and phosphate (Weishaar and Simpson, 1987). In this study, vitamin D-deficient diets with normal and high calcium/phosphate content are used to isolate the direct versus indirect effects of vitamin D deficiency on deep tissue sensitivity in rats.

Our findings show that 4 weeks of a vitamin D-deficient diet produces muscle mechanical hypersensitivity and balance deficits.

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Correspondence should be addressed to Peter G. Smith, University of Kansas Medical Center, Institute for Neurological Disorders, 3901 Rainbow Boulevard, Kansas City, KS 66160. E-mail: psmith@kumc.edu.

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Instead of correcting this phenotype, increased dietary calcium accelerated its development. Concurrent with muscle hypersensitivity was skeletal muscle hyperinnervation by putative nociceptor axons. This appears to be due to direct actions of vitamin D metabolites on rapid response VDRs, as both $1,25(OH)_2D3$ and the rapid response specific agonist 6-*s*-*cis*-lumisterol (JN) regulated neurite outgrowth by peripherin-positive sensory neurons in culture. The greater density of "pain-sensing" nerves in muscles of vitamin D-deficient rats may therefore contribute to the observed hypersensitivity.

Materials and Methods

Animals and diets

Animal protocols and procedures were in accordance with NIH guidelines for the care and use of laboratory animals and approved by the University of Kansas Medical Center Animal Care and Use Committee. Fifteen weaned female Sprague Dawley rats (Harlan) housed 2–3/cage in forced-air-ventilated micro-isolation cages with a 14 h light/10 h dark cycle and fed *ad libitum* normal chow. At 31 d of age, rats were anesthetized with ketamine 70 mg/kg (Pfizer)/xylazine 6 mg/kg (i.p.) (Lloyd Laboratories) and ovariectomized via bilateral hindflank incisions. Upon waking and at 24 and 48 h after the operation, rats were administered ketoprofen 5 mg/kg (s.c.) (Ketofen; Fort Dodge Animal Health) as an analgesic. Ovariectomized rats were used to eliminate estrous cycle-driven variations in behavioral sensitivity (Craft, 2007) and sensory neuronal VDR expression (Tague and Smith, 2011); this model also incorporates risk factors seen in affected human populations with musculoskeletal pain (female, postmenopausal or estrogen suppressed) (Gaugris et al., 2005; Alexander et al., 2007; Khan et al., 2010). The effectiveness of the ovariectomy was assessed at the end of the experiment by confirming that all uteri were atrophic.

At 48 d of age, rats were randomly assigned to treatment groups and fed one of three diets: Control: 2.2 IU/g vitamin D (cholecalciferol), 0.47% Ca, 0.3% P (Harlan Teklad, TD.07370); VD−/+Ca: vitamin D-depleted, 2.5% Ca, 1.5% P (Harlan Teklad, TD.07541); VD−: vitamin D-depleted, 0.47% Ca, 0.3% P (Harlan Teklad, TD.89123). The VD−/+Ca diet was based on previously published reports that increasing dietary Ca from 0.47% to 2.5% normalizes serum Ca in prolonged vitamin D deficiency (1.5% P is needed as a counterbalance) (Weishaar and Simpson, 1987). The identity of each diet was removed and replaced with a color code by an uninvolved technician to blind the investigators to the dietary treatments. Food was replaced twice weekly, and weighed before and after changing. Rats were maintained on their assigned diet for 4 weeks and weighed weekly. After 2 weeks, 1–2 ml of blood was drawn from the tail vein. Serum was collected and stored at −20°C. At the end of the study, rats were deeply anesthetized with ketamine 70 mg/kg/xylazine 6 mg/kg (i.p.), thoracic cavities were opened, blood was removed for serum analysis via cardiac puncture, and the rats were perfused with 50 ml of cold 0.9% saline containing 10 U/ml heparin (APP Pharmaceuticals) at a rate of 40 ml/min, followed by 150–200 ml of 4% formaldehyde, prepared in PBS from paraformaldehyde (Sigma-Aldrich).

Behavioral testing

All behavioral testing was performed in a quiet room under normal fluorescent lighting 6–8 h into the light cycle. The rat cages were moved to the behavior room 1 h before testing.

Muscle mechanical sensitivity. Humans with vitamin D deficiency exhibit muscle pain in muscles at multiple locations. However, the strongest association between vitamin D deficiency and pain is reported to occur in leg muscles (Heidari et al., 2010). Accordingly, we assessed sensitivity in hindlimb musculature. The muscle compression test we used is similar to one previously described (Skyba et al., 2005; Sharma et al., 2009). A digital Randall–Selitto instrument (IITC Life Science) with a plastic cap that has a 0.5-cm-wide surface area was used. During the week before diet manipulation, rats were habituated to rat slings (IITC Life Science) for 5 min on 2 separate days. One day before starting diets and each week thereafter, rats were stabilized in slings to allow free access to their hindlimbs. While holding the left hindpaw, the flat side of the probe was placed against the left inner calf and the pressure sensor was placed on the center of the exterior calf muscle. The instrument was slowly compressed until the rat attempted a limb withdrawal. In two instances occurring in different treatment groups, rats vocalized instead of withdrawing; compression was stopped to avoid tissue damage and force was recorded. These values were similar to those of other rats within the same group and were included in analysis. The calf was compressed three times and the maximum force applied was recorded and averaged. The left calf was shaved each week 3 d before muscle compression under brief isoflurane anesthesia. In a pilot study, we found that 2.5% lidocaine/2.5% prilocaine cream (TOLMAR) applied to the calf skin of control rats for 30 min prevented a withdrawal response to pinprick, but did not affect withdrawal responses to muscle compression (360 ± 65 g pretreatment vs 329 ± 43 g after treatment). This is consistent with previous findings (Skyba et al., 2005) and supports the idea that the cutaneous contribution to the compression withdrawal response is minimal. Muscle compression was analyzed before and after cutaneous anesthesia at week 4.

Cutaneous mechanical sensitivity. Cutaneous mechanical sensation was measured using methods similar to those previously described (Christianson et al., 2007). Rats were allowed to habituate for 20 min on a wire-mesh grid with Plexiglas dividers. A 4 g monofilament was applied to the center of the plantar surface five times per trial for a total of three trials. The number of positive responses was recorded, and the percentage withdrawal response for each paw was calculated. A response was considered positive when the rat retracted its hindpaw in response to the monofilament. The results shown are averages from the left and right hindpaws. Cutaneous sensitivity was measured 2 d before dietary manipulation and each week thereafter. In addition, we measured withdrawal threshold in all groups at week 4 using an electronic von Frey anesthesiometer (IITC Life Science). The results presented are averages from three trials on each foot.

Balance. Balance was assessed using methods similar to those previously described (Muller et al., 2008). An elevated, 2.5-cm-diameter round dowel was suspended between two support stands with an enclosed plastic platform at one end. At the ages of 28, 39, and 41 d, rats were trained by placing them on the beam at increasing distances from the platform box until they voluntarily walked the full 110 cm of the beam. Two days before starting the diets and at weeks 2 and 4, rats were videotaped for three beam walk trials. The videos were analyzed to determine the number of hindpaw footfall slips (faults) per beam walk, which were then averaged.

Muscle strength. Grip strength was measured using methods previously described (Smittkamp et al., 2010). Three days before starting diets and at weeks 2 and 4, rats were placed on a wire grid attached to a digital force gauge (San Diego Instruments). The maximum grip force applied by the forelimbs was measured as the rats were gently pulled until their grip was broken. This was repeated three times for each rat and the maximum force was recorded.

General locomotor activity. Rats were placed in a Force Plate Actimeter (BASi) (Fowler et al., 2001) and allowed to move freely for 5 min. Movements were detected by force transducers and recorded by a computer, and rearing events were recorded by the investigator.

Serum measurements

Serum 25-hydroxyvitamin D was measured by direct ELISA (Immunodiagnostik) according to manufacturer instructions. Serum calcium and phosphorous were measured by an outside laboratory (Physicians Reference Laboratory) by spectrophotometry.

Tissue processing

The left calf muscles (gastrocnemius and soleus) were postfixed in Zamboni's fixative overnight at 4°C. Weight and volume (determined by fluid displacement in a graduated cylinder) were recorded. The muscles were washed and cryoprotected at 4°C in PBS with 30% sucrose for 2 weeks. The entire calf was cut in the transverse plane at 25%, 50%, and 75% of the way through the muscle. The muscle pieces were embedded in tissue freezing media (Electron Microscopy Science), frozen in liquid nitrogen-cooled isopentane, and stored at −80°C. Thawed 20 μm cryosections were stained with hematoxylin and eosin (H&E).

The left tibia was postfixed in Zamboni's fixative overnight at 4°C. The bones were washed at 4°C in PBS changed daily for 3 d, then decalcified in PBS containing 10% EDTA, pH 7.4, for 2 weeks at 4°C. The decalcified

bones were cryoprotected overnight at 4°C in 30% sucrose. The bone was cut in half along the transverse plane. The proximal portion was cut in half along the sagittal plane and the medial portion was embedded in tissue freezing media for sectioning of the epiphyseal plate at 20 μm. The distal half of the bone was embedded in tissue freezing media for transverse sections of the diaphysis. Bones were frozen on dry ice, stored at −80°C, and sections were stained with H&E.

The right footpad was removed with a scalpel after saline perfusion and before perfusion with Zamboni's fixative. Footpads were fixed in Zamboni's fixative for 1.5 h at room temperature, washed, and cryoprotected at 4°C in 30% sucrose changed daily for 1 week. Footpads were bisected along the sagittal axis, embedded in tissue freezing media, frozen on dry ice, and stored at −80°C.

Immunostaining of nerve fibers in footpads and muscle

Thawed sections were preincubated in 1.5% donkey serum (Jackson ImmunoResearch Laboratories), 0.5% gelatin (Sigma-Aldrich), and 0.5% Triton X-100 (Sigma-Aldrich) prepared in Superblock (Thermo Scientific) for 1 h at room temperature, incubated overnight at room temperature in primary antibodies diluted in incubation solution (50% preincubation solution, 50% Superblock), and for 2 h with secondary antibodies. Slides were washed in PBS containing 0.25% Triton X-100 before and after the secondary antibody application. Primary and secondary antibodies were diluted as follows: 1:500 chicken anti-peripherin (Millipore Bioscience Research Reagents) (Tague and Smith, 2011), 1:200 sheep anti-calcitonin gene-related peptide (CGRP, Biomol) (Ruscheweyh et al., 2007; Tague and Smith, 2011), 1:1000 rabbit anti-vesicular monoamine transporter 2 (VMAT2, Millipore Bioscience Research Reagents) (Witkovsky et al., 2004), 1:1500 rabbit anti-Neurofilament H (NFH, Sigma) (Besalduch et al., 2011), 1:1200 rabbit anti-protein gene product 9.5 (PGP 9.5 Serotec) (Chakrabarty et al., 2011), 1:1500 donkey anti-chicken DyLight 488 (Jackson ImmunoResearch Laboratories), 1:750 donkey anti-sheep Dylight 649 (Jackson ImmunoResearch Laboratories), 1:1000 donkey anti-rabbit Alexa 647 (Invitrogen), or 1:200 donkey anti-rabbit Cy2 (Jackson ImmunoResearch Laboratories). α-Bungarotoxin Alexa Fluor 488 (Invitrogen) was applied during secondary incubation. All primary antibodies have been previously characterized as indicated. In addition, primary omission controls were performed.

Quantitation of innervation density

Innervation density was determined in deidentified, randomly sorted samples. Subject identity was revealed only at the completion of quantification. Nerve fibers included in analysis had signal intensities that were at least fourfold above background tissue fluorescence.

Muscle. Images were obtained from transverse sections of the left lateral gastrocnemius muscle, which is located on the outside of the calf where the pressure sensor was applied during muscle compressions. A total of 18 images were taken per animal, six each from three levels at 25%, 50%, and 75% through the proximodistal extent of the muscle. A stereological grid was superimposed over the images, and numbers of grid intersections overlying immunofluorescently labeled axons/neuromuscular junctions were counted and divided by the total number of tissue intersections to provide the apparent percentage area occupied by nerves/neuromuscular junctions (Clarke et al., 2010). Each image had a total field area of 1.3 mm^2, and each grid square area was 1720 μm^2.

Footpads. Three images were taken by a blinded observer from each of two sagittal sections ~100 μm on either side of the midline, which corresponds to roughly the same region used for cutaneous mechanical sensitivity testing. Intra-epidermal nerve fiber density (IENF) was determined by counting the number of immunofluorescently labeled nerves crossing the dermal–epidermal junction per millimeter, excluding fragments not crossing the dermal–epidermal junction (Lauria et al., 2005). To obtain total epidermal nerve fiber density, a stereological grid was superimposed over the images, and numbers of grid intersections overlying immunofluorescently labeled axons were counted and divided by the total number of epidermal intersections (excluding the keratinized layer, which is not innervated), to provide the apparent percentage of epidermal area occupied by nerves (Clarke et al., 2010). Each image had a total field area of 0.3 mm^2, and each grid square area was 112.5 μm^2.

Bone measurements

Bone sections (20 μm) were stained with hematoxylin and eosin and analyzed. For growth plate analysis, height and cell number of three fully visible chondrocyte columns in the center of each section were measured and averaged. Bone circumference and bone marrow area were measured in transverse sections of the diaphysis.

Primary neuronal cell cultures

Sensory. DRGs were removed from 6-week-old female Sprague Dawley rats, dissociated with 2 U/ml dispase (ICN Biomedicals) and 2 mg/ml collagenase type 1A (Sigma) and plated on glass coverslips (Bellco) coated with poly-D-lysine (Sigma) and laminin (Invitrogen) in 24-well culture dishes. Cultures were maintained for 4 d in Neurobasal A media (Invitrogen) containing 0.5 mM glutamine (Sigma), 2% B27 (Invitrogen), 100 μg/ml Primocin (InvivoGen), 20 μM FrdU (Sigma) and 20 μM uridine (Sigma), with concentrations of $1,25(OH)_2D3$ or JN that ranged from 0 to 100 pM. Cultures were incubated at 37°C with 5% CO_2 and media was changed after 24 and 72 h. Cells were fixed in 4% formaldehyde for 1 h, DAPI stained, and immunostained for peripherin (chicken 1:1000, Millipore Bioscience Research Reagents) (Tague and Smith, 2011), NFH (mouse 1:200, Sigma) (Herde et al., 2010), or VDR (rabbit 1:25, GeneTex) (Tague and Smith, 2011), peripherin, and growth-associated protein 43 (GAP43) (mouse 1:240, Zymed) (Watanabe et al., 2009). Culture studies were conducted in triplicate and repeated twice.

Sympathetic. Superior cervical ganglia were removed from 1-d-old rats, dissociated with 0.25% trypsin-EDTA (Invitrogen) and collagenase 1A (Sigma), and plated on poly-D-lysine and laminin-coated coverslips (Clarke et al., 2010). Cultures were maintained in Neurobasal A media as described above with the addition of 10 μg/ml NGF (Alomone Labs) for 48 h, with 0, 20, or 100 pM $1,25(OH)_2D3$. Cultures were fixed in 4% formaldehyde, DAPI stained, and immunofluorescently labeled for peripherin and VDR. Cultures were grown in triplicate and repeated twice.

Neurite area quantitation

A stereological grid was superimposed over culture images, and numbers of grid intersections crossing an immunostained nerve fiber were counted and divided by the total number of intersections within each field to provide neurite area; this was divided by the number of viable neurons per field, determined by DAPI staining, to provide an estimate of neurite area per neuron (Chakrabarty et al., 2008). Each image had a total field area of 0.15 mm^2, and each grid square area was 222.8 μm^2.

Statistical analysis

All values are expressed as mean ± SEM. One-way ANOVA was used to analyze results when comparing multiple treatments at a single time point. When comparing effects on animals over multiple time points with multiple treatments, two-way repeated-measures ANOVA was used. When data failed either the Shapiro–Wilk normality ($p < 0.05$) or Levene median equal variance ($p < 0.05$) tests, ANOVAs were conducted using the Kruskal–Wallis test for one-way ANOVA or Conover test for two-way ANOVA. *Post hoc* multiple comparisons were completed using the Student–Newman–Keuls method. For all tests, n equals 5 for each treatment group, except the cultures, where n equals 6 for each treatment group, and statistical significance was accepted at $p \leq 0.05$.

Results

A rat model of Vitamin D deficiency

Vitamin D deficiency in people occurs as a result of insufficient sunlight exposure and dietary limitations. In the laboratory rat, diet is the primary source of vitamin D and a diet depleted of vitamin D produces conditions that resemble deficiencies in humans. Groups of Sprague Dawley rats were fed one of three diets: Control: normal vitamin D, calcium, and phosphate; VD−/+Ca: vitamin D-depleted, elevated calcium and phosphorus; or VD−: vitamin D-depleted, normal calcium and phosphorus (Table 1). Rats fed vitamin D-deficient diets showed weight gains comparable to control subjects over the 4 week study (Table 1). However, food consumption was increased in the VD−/+Ca group

Table 1. Diet, weight, and serum analysis

	Diet			Food intake		Weight			Serum 25(OH)D		Serum Ca	Serum P
	Vitamin D3 (IU/g)	Ca (%)	P (%)	Week 2 (kcal/d)	Week 4 (kcal/d)	Week 0 (g)	Week 2 (g)	Week 4 (g)	Week 2 (nmol/L)	Week 4 (nmol/L)	Week 4 (mg/dl)	Week 4 (mg/dl)
Control	2.2	0.47	0.3	58.9 ± 0.4	63.7 ± 0.1	164 ± 4	224 ± 5	264 ± 5	51.8 ± 6.0	70.8 ± 6.3	11.0 ± 0.1	8.5 ± 0.4
VD−/+Ca	—	2.5	1.5	64.1 ± 1.3*	67.8 ± 1.9*	168 ± 4	225 ± 6	262 ± 9	24.6 ± 5.5*	6.6 ± 0.7*	11.0 ± 0.1	8.3 ± 0.4
VD−	—	0.47	0.3	59.9 ± 0.3	66.4 ± 0.6*	166 ± 3	224 ± 2	260 ± 5	18.3 ± 2.5*	4.2 ± 1.5*	11.0 ± 0.1	8.8 ± 0.4

*$p < 0.05$ compared to control. Values shown are means ± SEM.

(week 2 $p = 0.002$, week 4 $p = 0.028$) and in the VD− group at week 4 ($p = 0.014$) compared to controls (Table 1). Serum 25(OH)D concentrations in both VD−/+Ca and VD− rats were reduced below 25 nmol/L by week 2 and below 10 nmol/L by week 4 (Table 1, $p < 0.001$ for both groups at weeks 2 and 4 compared to control). Serum Ca and P levels at week 4 were not reduced, even though Ca is reported to be decreased with longer duration of vitamin D deficiency (Weishaar and Simpson, 1987). Therefore, dietary vitamin D restriction in rats produced a selective deficiency in serum 25-hydroxyvitamin D levels, similar to that seen in humans.

Vitamin D-deficient rats show skeletal muscle hypersensitivity

To determine whether increased muscle sensitivity reported in vitamin D-deficient humans also occurs in our rodent model, we assessed behavioral responses to calf muscle compression using a modified Randall–Selitto testing device. In control rats, force required to elicit a withdrawal response increased between 0 and 2 weeks, consistent with increasing body mass (Table 1), and plateaued thereafter (Fig. 1*A*). In VD− rats, mechanical sensitivity was normal through week 2, but was increased by week 4 ($p = 0.022$, Fig. 1*A*). Rats receiving the vitamin D-deficient diet with elevated Ca showed normal sensitivity at week 1, but were markedly hypersensitive at week 2 and thereafter (Fig. 1*A*; week 2: $p = 0.022$, VD−/+Ca vs control; $p = 0.043$, VD−/+Ca vs VD−; week 3: $p = 0.049$, VD−+Ca vs control; week 4: $p = 0.04$, VD−/+Ca vs control). Thus, the high-calcium diet not only failed to reverse the hyperalgesic phenotype, but actually accelerated the onset of deep tissue mechanical hypersensitivity. Application of topical anesthetic cream at week 4 did not affect withdrawal threshold in vitamin D-deficient rats (262 ± 29 g vs 292 ± 18 g for VD−/+Ca; 266 ± 18 g vs 277 ± 22 g for VD−).

Vitamin D deficiency does not alter cutaneous sensitivity

We asked whether vitamin D deficiency leads to generalized hypersensitivity. Accordingly, we evaluated mechanical sensitivity of the hindpaw plantar cutaneous surface using a calibrated monofilament throughout the study. There were no differences between groups in mechanical withdrawal responses at any time (Fig. 1*B*). We further assessed cutaneous sensitivity by determining withdrawal threshold at week 4 by using an electronic von Frey anesthesiometer. There were no significant differences between groups (Control, 60 ± 4 g; VD−/+Ca, 51 ± 3 g; VD−, 57 ± 5 g). Therefore, vitamin D deficiency does not result in generalized changes in sensory thresholds, as evidenced by the lack of changes in cutaneous mechanical withdrawal thresholds.

Balance is impaired in Vitamin D deficiency

Falls occur more frequently in vitamin D-deficient individuals (Annweiler et al., 2010), suggesting that balance may be impaired. We assessed balance in vitamin D-deficient rats using a beam-walk test where numbers of foot slips were measured from

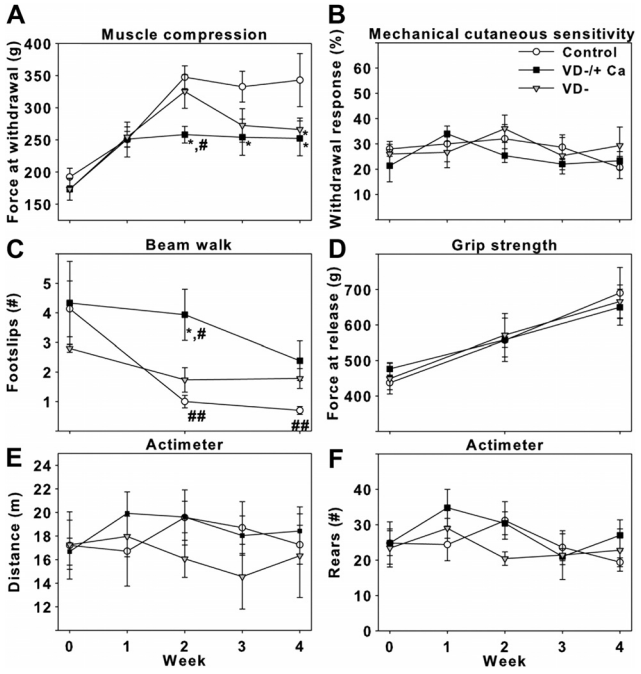

Figure 1. Behavioral comparisons of control (○), VD−/+Ca (■), and VD− (▽) rats. ***A***, Changes in deep tissue pressure sensitivity, measured as the maximum force (g) required to elicit an attempted limb withdrawal or vocalization to mechanical compression of the calf muscle. ***B***, There were no changes in cutaneous mechanical sensitivity as measured by the percentage of withdrawal responses to the application of a 4 g monofilament to the plantar surface of the hindpaw. ***C***, Changes in balance were measured by the number of hindpaw slips while traversing an elevated 2.5-cm-diameter, 110-cm-long beam. VD− and VD−/+Ca rats failed to improve, while control rats improved compared to initial performance. ***D***, There were no changes in forelimb grip strength, as measured by the force (g) required to break the rats grip. ***E***, ***F***, Assessment of locomotor activity during a 5 min recording using a force-plate actimeter revealed no changes in distance traveled (***E***) or number of rearing events (***F***). *$p < 0.05$ compared to control in same week, #$p < 0.05$ compared to VD− in same week, ##$p < 0.5$ compared to week 0 within the same group.

video recordings. Performance improved between 0 and 2 weeks in rats receiving a normal diet, and slips remained low at 4 weeks (Fig. 1*C*; $p \leq 0.001$ for week 0 vs week 2 and week 4). Rats receiving the VD− diet, however, failed to improve throughout the study. Rats receiving the VD−/+Ca diet performed worse than controls at weeks 2 and 4 ($p = 0.002$ and 0.024, respectively) and worse than VD− at week 2 ($p = 0.017$). Because vitamin D-deficient rats tended to sit lower on the beam, use more caution, and make more balance corrections, the balance deficit may be greater than our assessments indicate.

Vitamin D deficiency does not impair mobility or muscle strength

Many factors could contribute to impaired beam-walk performance in vitamin D-deficient rats, including myopathic changes known to occur in protracted vitamin D deficiency (Schott and

Wills, 1976). To assess whether overt muscle dysfunction occurs in our model of vitamin D deficiency, we measured grip strength (Fig. 1*D*). Forelimb grip strength increased with age in all groups, and vitamin D-deficient rats showed no deficits relative to rats on control diet, consistent with maintenance of normal muscle function. To determine whether the apparent balance deficits were associated with diminished mobility, the pattern of exploratory locomotor activity of each rat was analyzed in a force-plate actimeter. There were no significant differences in the overall activity between treatment groups, including total distance traveled (Fig. 1*E*) and number of rearing events (Fig. 1*F*). Thus, the balance deficits observed in our vitamin D-deficient rats do not appear to result from grossly impaired locomotor mobility or overt muscle weakness.

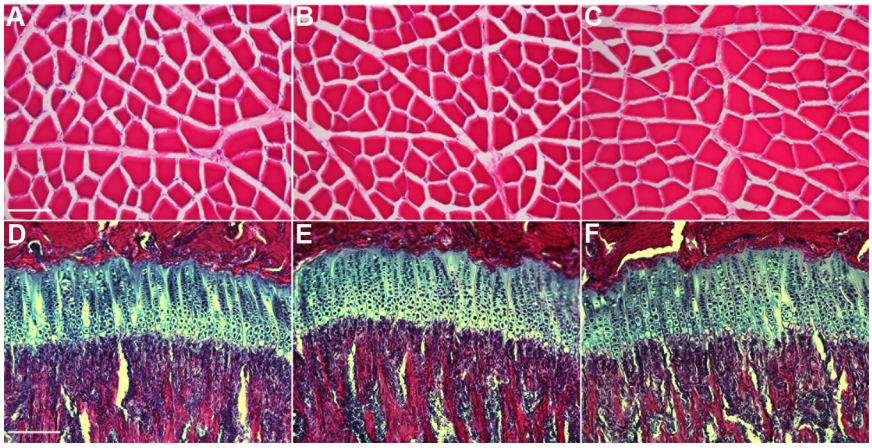

Figure 2. Images of hematoxylin and eosin-stained lateral gastrocnemius muscle and tibial growth plate sections. ***A–C***, Hematoxylin and eosin-stained transverse sections of the lateral gastrocnemius of rats receiving control (***A***), VD−/+Ca (***B***), or VD− (***C***) diets. There were no discernible differences between groups. Scale bar, 100 μm for ***A–C***. ***D–F***, Sagittal sections (20 μm) of tibial growth plates from rats receiving control (***D***), VD−/+Ca (***E***), or VD− (***F***) diets. There were no discernible differences between groups. Scale bar, 20 μm for ***D–F***.

Deep tissue hypersensitivity occurs in the absence of gross muscle atrophy or bone pathology

Because vitamin D deficiency and deep muscle hypersensitivity are well established at 4 weeks after initiating vitamin D-deficient diets, we examined tissues at this time. Calf muscle volume (see Fig. 3*A*) and weight (data not shown) were essentially identical in all groups, and there were no discernible histological differences in muscle sections between groups (Fig. 2*A–C*), corroborating measurements of muscle strength and confirming an absence of muscle atrophy. It has been suggested that musculoskeletal pain in vitamin D deficiency derives from an expanding uncalcified bone matrix exerting pressure on the richly innervated periosteum (Mascarenhas and Mobarhan, 2004). In fact, shortened and bent tibia with enlarged and disorganized epiphyseal growth plates reportedly occur with prolonged vitamin D deficiency even when serum calcium is normal (Lester et al., 1982). We therefore examined tibiae from our vitamin D-deficient rats and found no overt abnormalities or differences in size or shape relative to controls. Histological analyses of decalcified tibial sections also showed organized growth plates with no expansion, either in height or number of chondrocytes per cartilage column (Table 2, Fig. 2*D–F*). Similarly, we did not observe changes in diaphysis circumference or marrow area (Table 2). These findings comport with prior observations that musculoskeletal pain in vitamin D deficiency occurs before bone pathology (Masood et al., 1989) and suggest that gross changes in muscle or bone integrity do not underlie deep muscle pain in the early stages of vitamin D deficiency.

Table 2. No changes in bone parameters were found in rats fed vitamin D-deficient diets for 4 weeks

	Growth plate		Diaphysis	
	Thickness (μm)	Cell #/column	Circumference (mm)	Marrow area (mm^2)
Control	282 ± 20	20.8 ± 0.9	6.9 ± 0.1	3.5 ± 0.1
VD−/+Ca	274 ± 25	20.0 ± 1.0	7.1 ± 0.4	3.6 ± 0.3
VD−	279 ± 14	21.1 ± 1.0	6.8 ± 0.4	3.5 ± 0.2

No significant differences were found. Values shown are mean ± SEM.

Vitamin D deficiency causes muscle hyperinnervation by unmyelinated CGRP-ir axons

There is growing evidence that many clinical pain syndromes are accompanied by nociceptor axon sprouting within affected peripheral tissues (Bohm-Starke et al., 1999; Alfredson et al., 2003; Schubert et al., 2005). Because steroid hormones other than vitamin D can influence sensory axonal outgrowth (Blacklock et al., 2005), we examined skeletal muscle innervation in control and vitamin D-deficient rats. Sections were immunostained for peripherin, a marker used to identify intact small-diameter unmyelinated axons (Goldstein et al., 1991). Peripherin-immunoreactive (-ir) nerve density was increased nearly twofold

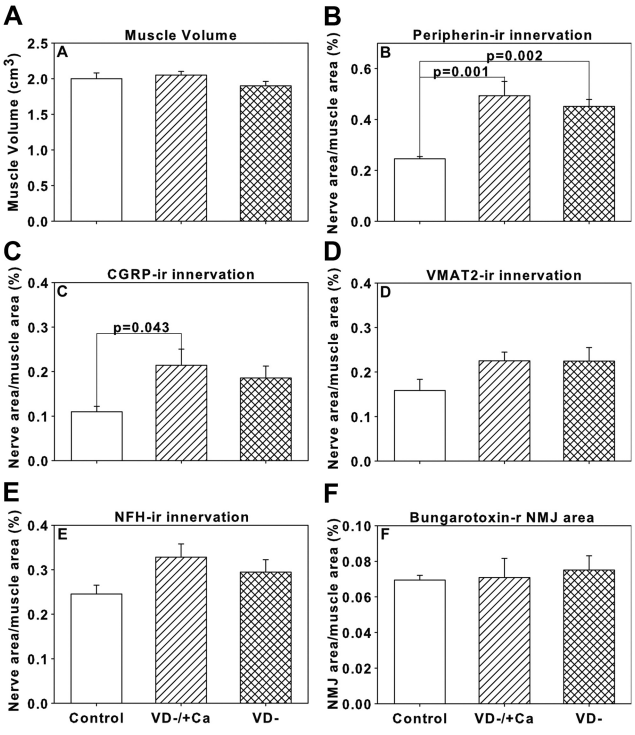

Figure 3. Comparisons of innervation densities of the lateral gastrocnemius muscles of rats after 4 weeks on control, VD−/+Ca, or VD− diets. ***A***, There were no changes in the volumes of the calf muscles as measured by fluid displacement. ***B–E***, There were significant increases in the percentage of nerve area per field area of peripherin-immunoreactive (ir) fibers (***B***) and CGRP-ir sensory peptidergic nerves (***C***) in muscles from vitamin D-deficient rats, but not VMAT2-ir sympathetic (***D***) or NFH-ir myelinated (***E***) nerve fibers. ***F***, There were also no changes in neuromuscular junction (NMJ) area as measured by α-bungarotoxin binding reactivity (−r).

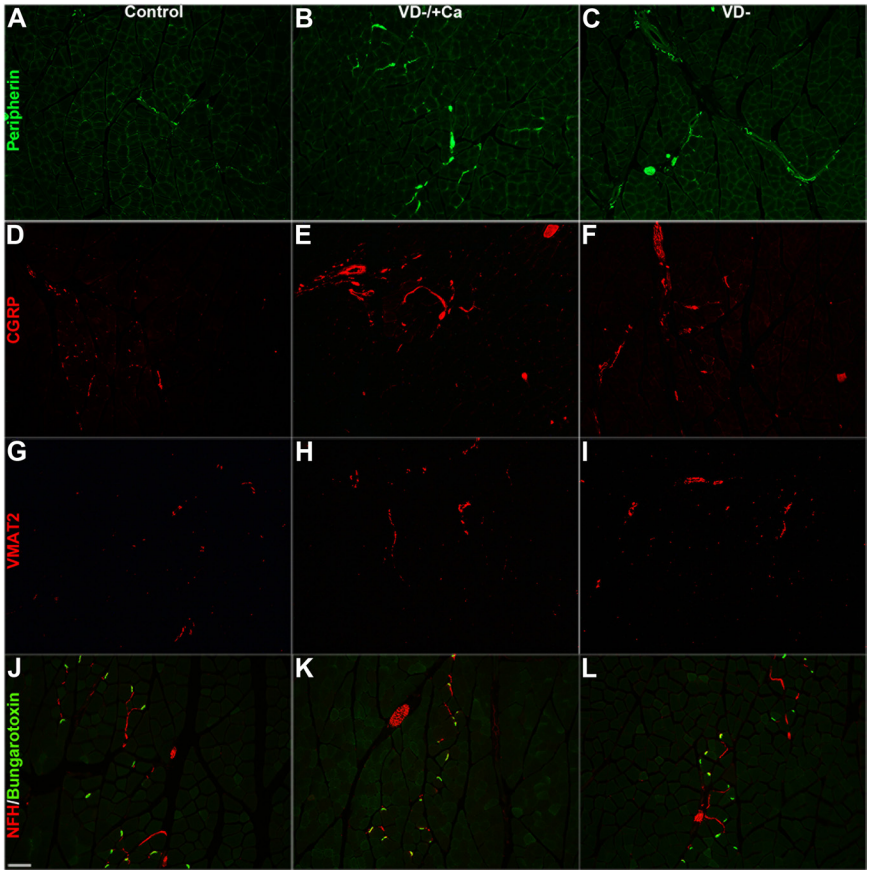

Figure 4. Immunoreactive nerves in gastrocnemius muscle. Transverse sections of the lateral gastrocnemius of rats receiving control (***A***, ***D***, ***G***, ***J***), VD−/+Ca (***B***, ***E***, ***H***, ***K***), or VD− (***C***, ***F***, ***I***, ***L***) diets were immunostained for peripherin (***A–C***, green), CGRP (***D–F***, red), VMAT2 (***G–I***, red), or NFH (***J–L***, red) and bungarotoxin (***J–L***, green). Scale bar (in ***J***), 50 μm for all panels.

in VD−/+Ca ($p = 0.001$) and VD− ($p = 0.002$) relative to control rats (Figs. 3*B*, 4*A–C*). To identify which populations of fibers are increased in vitamin D deficiency, muscle sections were immunostained for CGRP as a marker for "peptidergic" nociceptors (Lawson, 1992), and VMAT2 for noradrenergic sympathetic axons (Headley et al., 2007). Approximately 90% of all CGRP-ir neurons contained peripherin regardless of treatment (Control, 87 ± 2%; VD−/+Ca, 91 ± 3%; VD−, 93 ± 5%). CGRP-ir axon density was increased approximately twofold (Figs. 3*C*, 4*D–F*; $p = 0.043$, VD−/Ca vs control), while that of VMAT2-ir was not significantly different (Figs. 3*D*, 4*G–I*). To determine whether myelinated fibers innervating the muscle are affected in vitamin D deficiency, muscle sections were immunostained for neurofilament H (NFH), a marker of large myelinated motor and proprioceptive fibers, and α-bungarotoxin, which reveals terminal muscle innervation by staining motor end plates. We found no significant changes in sectional area of NFH-ir (Figs. 3*E*, 4*J–L*) or bungarotoxin between control and vitamin D-deficient muscles. These findings indicate that vitamin D deficiency leads to selective muscle hyperinnervation by putative peptidergic nociceptors.

Cutaneous innervation density is not altered by vitamin D deficiency

To determine whether vitamin D deficiency also leads to hyperinnervation of cutaneous tissues, we examined epidermal innervation of hindpaw footpads. In humans, changes in intra-epidermal nerve fiber density are most commonly measured in skin biopsies by counting the number of nerve fibers that cross the dermal–epidermal junction using the pan-neuronal marker PGP 9.5 (Lauria et al., 2005) (Fig. 5*A–C*). Using this method, we found no significant differences in intra-epidermal nerve density (Fig. 5*G*). However, this method does not account for potential growth or branching of axons within the epidermis. We therefore also examined the total density of PGP 9.5-ir nerve fibers within the epidermis, but still found no significant differences between groups (Fig. 5*I*). Because PGP 9.5 is a pan-neuronal marker and the majority of nerve fibers in the epidermis are not sensory peptidergic nerve fibers, changes in peptidergic nerve fiber density could have been masked. We therefore also examined CGRP-ir epidermal nerve fibers (Fig. 5*D–F*), but still found no differences using either method of quantitation (Fig. 5*H*,*J*), suggesting that 4 weeks of a vitamin D-deficient diet does not lead to changes in epidermal innervation, which is consistent with the lack of observed changes in cutaneous sensitivity.

Sensory growth cones are enriched in VDR protein

Adult sensory DRG neurons *in vivo* contain receptors and metabolic enzymes for vitamin D (Tague and Smith, 2011). Accordingly, vitamin D deficiency could affect muscle innervation through a direct action on neurons. To test this hypothesis, adult DRG neurons were grown *in vitro*. We first verified VDR expression in cultured neurons by immunostaining, and observed VDR-ir localized to the nucleus and somal cytoplasm (Fig. 6*A*,*B*). Interestingly, VDR appeared to be concentrated in growth cones of peripherin-ir neurites, where it extended distally into filopodia and showed spatial colocalization with the growth-associated protein GAP43 (Fig. 6*C–E*), suggesting a role for VDR in growth cone kinetics.

1,25(OH)$_2$D3 modulates neurite outgrowth from putative nociceptor neurons

To determine whether the VDRs might be playing a role in axonal sprouting, we measured neurite outgrowth from peripherin-ir DRG sensory neurons cultured with various concentrations of 1,25(OH)$_2$D3. Normal mean serum concentration of 1,25(OH)$_2$D is ~80 pM (Lund et al., 1979), whereas a study of patients with severe musculoskeletal pain reported 1,25-dihydroxyvitamin D levels ranging from 12 to 45 pM (Gloth et al., 1991). To bracket this range, we added 1,25(OH)$_2$D3 in concentrations between 0 and 100 pM to primary DRG cultures. Peripherin-ir DRG neurons grown with concentrations of 1,25(OH)$_2$D3 between 60 and 100 pM, corresponding to normal serum levels, showed typical axon outgrowth (Fig. 6*A*). However, below 60 pM, there was an increase in neurite outgrowth, peaking at 20 pM, where outgrowth was 60% greater than controls (20 pM vs 0, 60, or 100 pM, $p < 0.001$; 20 pM vs 10 or 80 pM, $p = 0.002$; 20 pM vs 40 pM, $p = 0.011$; 40 pM vs 0 pM, $p = 0.048$) (Fig. 6*G*,*I*). These findings indicate that 1,25(OH)$_2$D3 can directly affect sensory axon outgrowth from small fiber nerves.

Vitamin D does not alter sympathetic neurite outgrowth

In vivo findings suggest that vitamin D's effects are restricted to unmyelinated sensory neurons. To confirm vitamin D's selectivity, we cultured superior cervical ganglion (SCG) sympathetic neurons. Immunostaining showed VDR-ir in SCG neuronal cytoplasmic and nuclear compartments (Fig. 7*A–C*). However, unlike sensory neurons, VDR-ir appeared to be uniform throughout sympathetic neurites and was not enriched in terminal axonal growth cones (Fig. 6*C*,*E*). Varying concentrations of $1,25(OH)_2D3$ also failed to affect sympathetic neurite outgrowth (Fig. 6*F*), consistent with observations that VMAT2-ir axon density did not change in vitamin D-deficient rats.

VDR rapid response mechanisms regulate sensory neurite outgrowth

VDRs can signal through either classical nuclear genomic binding sites or rapid response pathways via membrane-bound receptors that modulate ion flux and generation of second messengers (Mizwicki and Norman, 2009). The enrichment of VDR in growth cones of vitamin D-sensitive sensory axons, but not insensitive sympathetic axons, suggests that axon outgrowth may be regulated by nongenomic VDRs. To determine whether sensory axon outgrowth is regulated by rapid membrane signaling mechanisms, we used JN, which selectively binds to and activates membrane VDRs but has no effect on nuclear VDR signaling (Norman et al., 1997). JN was applied to DRG cultures in the same concentrations as $1,25(OH)_2D3$ in the previous experiment. Immunostaining for large-diameter NFH-ir neurons, which primarily express only nuclear VDRs *in vivo* (Tague and Smith, 2011), showed no effect of JN on outgrowth, indicating that large-diameter neurons fail to respond to low levels of membrane VDR activation with increased outgrowth (Fig. 6*J*). However, JN did elicit neurite outgrowth of peripherin-ir fibers at low concentrations similar to those of $1,25(OH)_2D3$, with peak outgrowth occurring at ~20 pM (Fig. 6*J*, 20 pM vs 0, 60, 80, or 100 pM, $p < 0.001$; 20 pM vs 40 pM, $p = 0.011$; 10 pM vs 60 pM, $p < 0.001$, 10 pM vs 0, 80, or 100 pM, $p = 0.001$; 10 pM vs 40 pM, $p = 0.025$). Therefore, low levels of rapid response VDR activation are most likely to enhance neurite outgrowth from putative nociceptors.

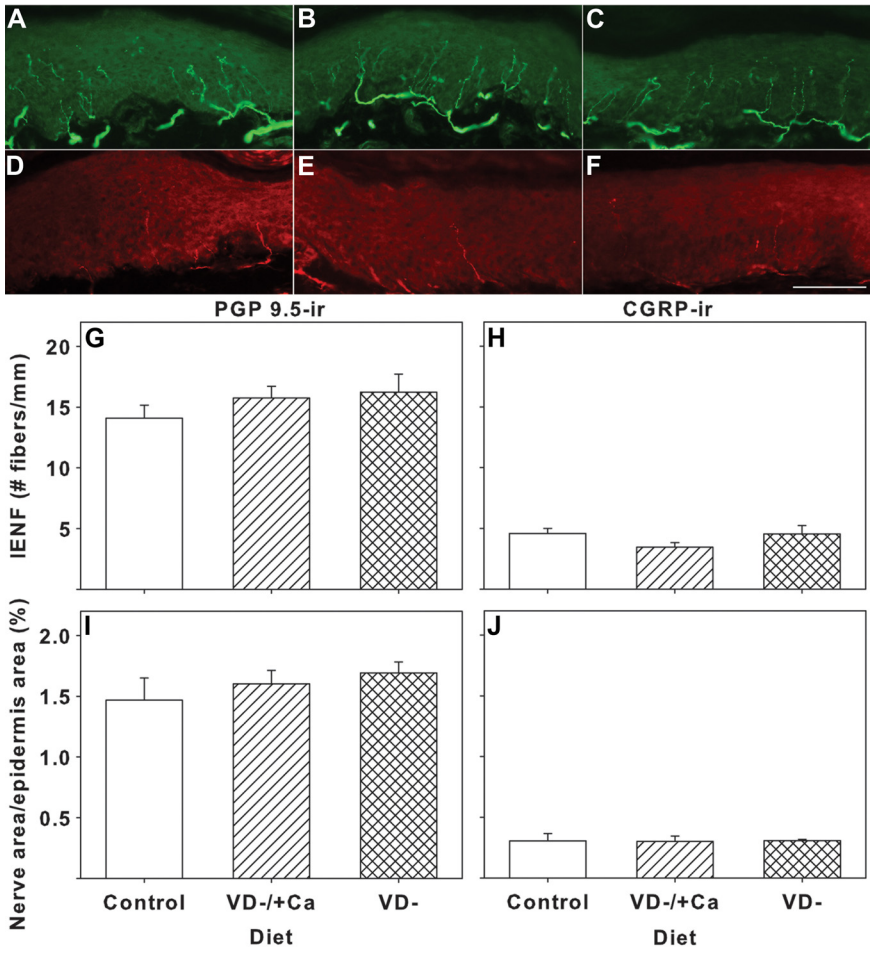

Figure 5. Comparisons of innervation densities of footpad epidermis in rats after 4 weeks on control, VD−/+Ca, or VD− diets. Sagittal sections of footpads from rats receiving control (***A***, ***D***), VD−/+Ca (***B***, ***E***), or VD− (***C***, ***F***) diets were immunostained for PGP 9.5 (***A–C***, green) or CGRP (***D–F***, red). There were no significant differences in the number of intra-epidermal nerve fibers per millimeter (***G***, ***H***, IENF; number of fiber nerves crossing the dermal– epidermal junction) or the total nerve fiber density (***I***, ***J***) within the epidermis in sections immunofluorescently labeled with PGP (***G***, ***I***) or CGRP-ir (***H***, ***J***). Scale bar (in ***F***), 100 μm in all panels.

Discussion

Chronic musculoskeletal pain is a widespread and costly disorder for which clinical therapy is relatively ineffective (Stewart et al., 2003; De Inocencio, 2004; Bergman, 2007). Some types of musculoskeletal pain (e.g., fibromyalgia) likely occur independent of vitamin D status (de Rezende Pena et al., 2010; Heidari et al., 2010), but a substantial body of clinical data implicates vitamin D deficiency in nonspecific musculoskeletal pain (Gloth et al., 1991; Glerup and Eriksen, 1999; Plotnikoff and Quigley, 2003; Macfarlane et al., 2005; Benson et al., 2006; de Torrenté de la Jara et al., 2006; Heidari et al., 2010; McBeth et al., 2010) and suggests that supplementation attenuates pain in some individuals (Gloth et al., 1991; Glerup and Eriksen, 1999; de Torrenté de la Jara et al., 2006; Heaney, 2008). Nonetheless, the role of vitamin D in musculoskeletal pain remains controversial in light of limitations inherent to human clinical studies.

Behavioral symptoms of early vitamin D deficiency, similar to those occurring in humans, are replicated in this rodent model, including deep muscle sensitivity and balance deficits. Protracted vitamin D deficiency results in major bone pathology, a proposed origin of musculoskeletal pain (Mascarenhas and Mobarhan, 2004). However, morphological analyses showed normal skeletal features after 4 weeks of a vitamin D-deficient diet, indicating that skeletal pathology is unlikely to contribute to muscle pain at this time. Moreover, simple muscle compression is unlikely to have exerted significant pressure on adjacent bone. While myopathic changes could also produce sensitivity, we found no evidence of muscle wasting or diminished muscle strength. Although our study was limited to the hindlimb musculature (the site most strongly linked to vitamin D-deficiency pain; Heidari et al., 2010), it is likely to apply to other muscles in which pain has been reported (Al Faraj and Al Mutairi, 2003; de Torrenté de la Jara et al., 2004; Lotfi et al., 2007; Heidari et al., 2010; McBeth et

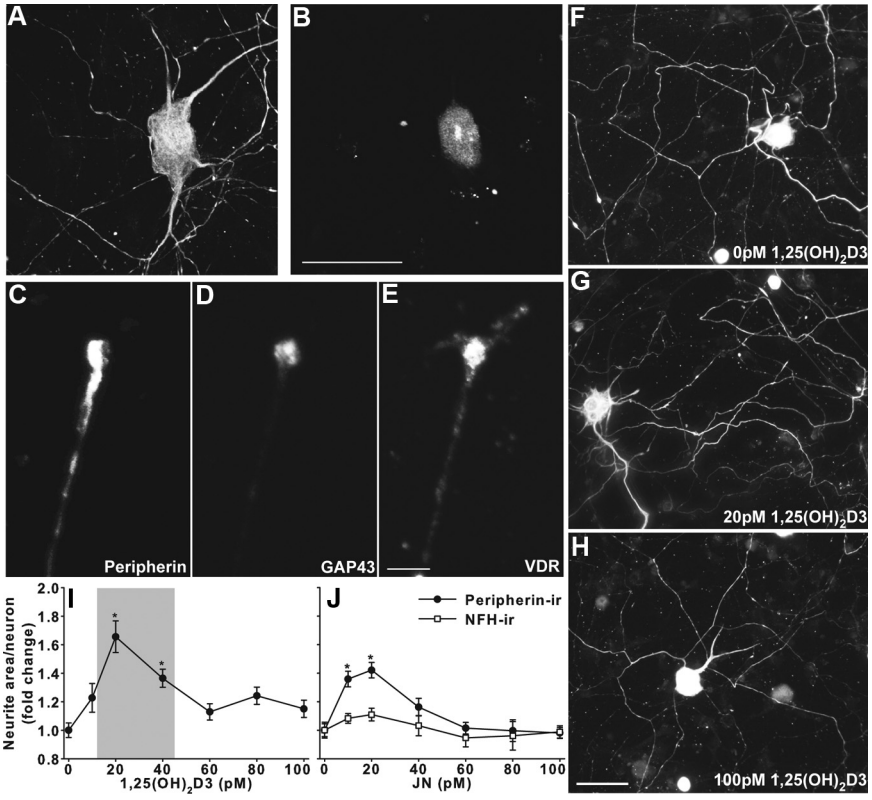

Figure 6. 1,25(OH)$_2$D3 acts directly on peripherin-ir sensory nerves to regulate axonal growth. ***A***, ***B***, Primary DRG cultures immunostained for peripherin (***A***) and vitamin D receptors (***B***, VDR), showing that VDR is expressed by cultured sensory neurons and localized to both the nucleus and the cytoplasm in cultures without added 1,25(OH)$_2$D3 (scale bar, 50 μm). ***C–E***, At a higher magnification, peripherin-ir neurites (***C***) contain the growth cone marker GAP43 (***D***) localized to the axon tip, and this colocalizes with VDR (***E***), which also extends into the filopodia. Scale bar (in ***E***), 5 μm for ***C–E***. ***F–H***, Representative images of neurons treated with 0 (***F***), 20 (***G***), or 100 pM (***H***) 1,25(OH)$_2$D3. Scale bar (in ***H***), 50 μm for ***F–H***. ***I***, Fold change in peripherin-ir neurite area per neuron at various 1,25(OH)$_2$D3 concentrations. Results reveal a biphasic curve with the peak at 20 pM. The shaded area corresponds to 1,25(OH)$_2$D3 levels reported in patients with severe pain (Gloth et al., 1991). ***J***, DRG cultures treated with JN, a selective agonist for membrane/cytoplasm-localized rapid response VDRs, and stained for peripherin (●) or NFH (□). While there was no significant change in NFH-ir neurite area /neuron, there was a biphasic response to JN, with a peak at 20 pM in peripherin-ir axons. $^*p < 0.05$.

al., 2010). The observations presented here lend strong credence to the idea that mild or early vitamin D deficiency can result in skeletal muscle pain in the absence of gross musculoskeletal pathology.

Our findings support the idea that muscle pain in vitamin D deficiency is directly attributable to diminutions of this secosteroid. A problem in human studies is the difficulty presented by confounding variables such as light exposure, body mass, hormonal fluctuations, and serum calcium levels. By using ovariectomized rats, the impact of hormonal variations was minimized. Similarly, changes in body mass can reasonably be excluded as a participating factor. An important variable that could contribute to both hypersensitivity and disequilibrium is serum calcium levels, which can affect muscle and nerve function. Prolonged vitamin D deficiency induces hypocalcemia by interfering with calcium homeostasis. Hypocalcemia can be prevented by elevating dietary calcium and phosphorus (Weishaar and Simpson, 1987), and one subject group in our study received such a diet. However, serum measurements showed normal calcium and phosphorous levels, despite significantly lower serum 25(OH)D levels at 2–4 weeks in both vitamin D-deficient groups. This is consistent with reports of normal serum calcium levels in patients with vitamin D deficiency and musculoskeletal pain (Masood et al., 1989; Gloth et al., 1991).

It was intriguing, however, that a high-calcium diet actually accelerated the onset of muscle hypersensitivity relative to rats receiving vitamin D-deficient diet alone. Elevated dietary calcium in rats is reported to inhibit the 1α-hydroxylase conversion of 25(OH)D to 1,25(OH)$_2$D (Mallon et al., 1981; Anderson et al., 2010), which may have exacerbated effects of the vitamin D-deficient diet. Whatever the mechanism, this finding may be germane considering that 43% of the population uses calcium supplements (Bailey et al., 2010). Calcium supplements taken to promote bone health might actually exacerbate muscle pain in vitamin D-deficient individuals.

Many factors may contribute to altered deep muscle sensitivity. While plasticity of central pathways may be the primary mediator of muscle pain in fibromyalgia (Bradley, 2009), local factors within the muscle itself are likely responsible for other types. Muscle pain is believed to derive from unmyelinated sensory fibers located within the surrounding fascia and discrete "pain spots" located deep within the muscle (Meadows, 1970). Our finding that the affected tissue shows selective hyperinnervation by putative nociceptors is in accord with reports describing hypersensitivity accompanied by abnormal increases in presumptive "pain-sensing" nerves (Bohm-Starke et al., 1999; Alfredson et al., 2003; Schubert et al., 2005). While the contribution of increased innervation to pain sensitivity remains unclear, actively growing axons show greater excitability than quiescent axons (Jänig et al., 2009), as do neurons with more complex axonal geometries (Janse et al., 1999). Similarly, more extensive axonal branching likely results in greater summation of locally evoked depolarizing potentials. Accordingly, the finding that low vitamin D levels induce sprouting from putative nociceptors is consistent with increased muscular sensitivity to mechanical stimulation.

Another factor that could contribute to increased sensitivity is muscle inflammation. Vitamin D has anti-inflammatory properties (Skyba et al., 2005; Baeke et al., 2010), so deficiency of this hormone could make tissues more susceptible to inflammation. However, histological analysis of muscle sections showed no evidence of immune cell infiltration in vitamin D-deficient rats, suggesting that muscle inflammation is not the primary cause of muscle hypersensitivity. Nonetheless, hyperinnervation by CGRP-ir axons may increase the propensity for neurogenic inflammation within muscle. Antidromic activation of peptidergic c-fibers results in release of CGRP and tachykinins, which promote vasodilation, protein extravasation, mast cell degranulation, and infiltration and activation of immune cells (Maggi, 1995). This can further increase CGRP and tachykinin production (Ambalavanar et al., 2006), resulting in the release of cytokines, growth factors, and other

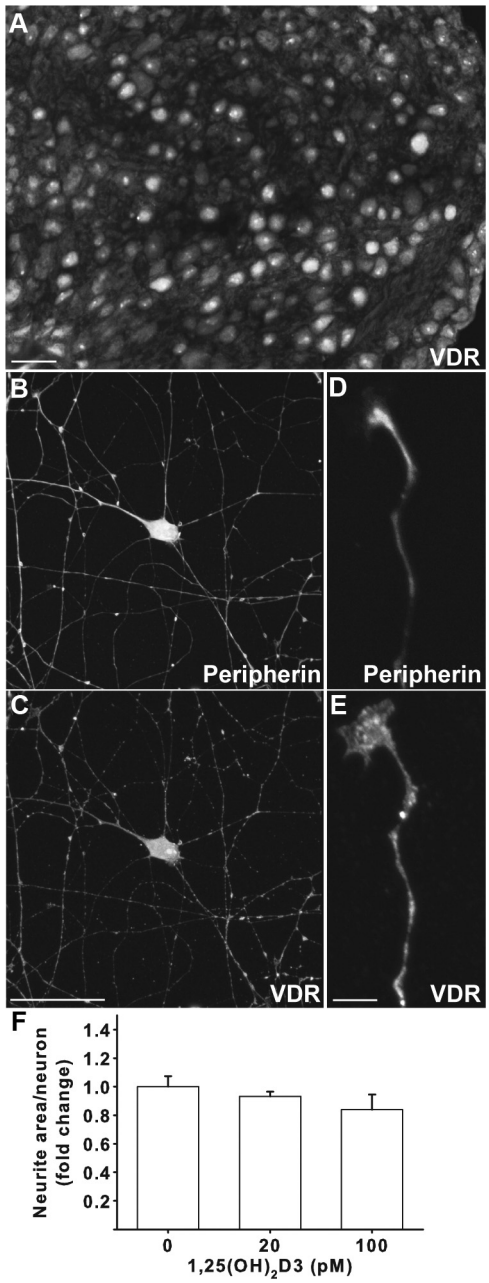

Figure 7. Sympathetic neurons express VDRs, but $1,25(OH)_2D3$ has no effect on neurite outgrowth. ***A***, Sympathetic neurons of SCGs were immunofluorescently labeled for VDR. VDR was localized to the nucleus and cytoplasm of selected neurons. ***B–E***, Primary SCG cultures immunofluorescently stained for peripherin (***B***, ***D***) and VDR (***C***, ***E***). Scale bars: (in ***B***) ***B***, ***C***, 50 μm; (in ***D***) ***D***, ***E***, 5 μm. VDR was localized throughout the cell body (***C***), neurite (***C***, ***E***), and growth cone (***E***). ***F***, Primary SCG cultures were grown for 48 h with 0, 20, or 100 pM $1,25(OH)_2D3$ and immunofluorescently labeled with peripherin. There were no significant changes in neurite area/neuron.

substances by the tissue, which can sensitize nociceptor terminals (Richardson and Vasko, 2002). Accordingly, while inflammation may not be a requirement for vitamin D-deficient skeletal muscle hypersensitivity and hyperinnervation, increased nociceptor fiber density could contribute to the establishment of chronic muscle pain through neurogenic inflammatory mechanisms.

The impaired beam walk performance in our rodent model is consistent with observations that falls are more frequent in vitamin D-deficient humans (Annweiler et al., 2010). However, the mechanism remains uncertain. While bone or muscle pathology could contribute, we failed to detect histopathological changes. Indices of motor activity and muscle strength were also normal, similar to findings that vitamin D-deficient humans have normal grip strength and gait speed (Faulkner et al., 2006). Another possibility is that vitamin D deficiency leads to proprioceptive deficits; however, myelinated axons, which include proprioceptors, do not appear to be altered in vitamin D deficiency. Certainly other aspects of peripheral or central proprioceptive pathways could well be affected. Alternatively, in light of the muscle hypersensitivity and hyperinnervation occurring in these animals, an equally plausible explanation is that muscle pain evoked during movement impairs the ability to make fine motor corrections, leading to increased balance beam foot faults.

An interesting feature of vitamin D deficiency-induced nociceptor hyperinnervation and hypersensitivity is its tissue selectivity; skeletal muscle showed both nociceptor hyperinnervation and hypersensitivity, but hindpaw skin showed neither. Subphysiological levels of $1,25(OH)_2D$ promote nociceptor neuron sprouting *in vitro* in the absence of target, indicating that vitamin D induces sprouting by acting directly on the neuron. However, other factors likely account for tissue selectivity. For example, all nociceptor populations may be affected, but some targets provide an environment permissive to sprouting (muscle) while others do not (skin). Similarly, local tissue concentrations of $1,25(OH)_2D$ may vary. Alternatively, perhaps only subpopulations of nociceptor neurons, such as those projecting to skeletal muscle, respond to reduced $1,25(OH)_2D$ by sprouting. Additional studies are needed to fully assess the mechanism of this specificity.

In neurons responding to hypovitaminosis D, there are several mechanisms by which $1,25(OH)_2D$ levels may affect axon outgrowth. Rapid response VDRs are known to regulate PKC, MAPK, PLC, PLA_2, Src, and Raf activation, Ca^{2+} and Cl^- channel opening, and sphingomyelin hydrolysis (Mizwicki and Norman, 2009). Any of these downstream mediators could regulate axon growth.

This study used ovariectomized rats, partially because women with reduced ovarian hormones (i.e., postmenopausal or estrogen-suppressed) are particularly susceptible to developing vitamin D deficiency and musculoskeletal pain (Gaugris et al., 2005; Alexander et al., 2007; Khan et al., 2010). In addition, it was thought that the reduction in VDR expression in sensory nerves following ovariectomy might enhance the effects of vitamin D deficiency (Tague and Smith, 2011). However, there are correlative data from clinical studies showing that men also develop hypovitaminosis D-induced musculoskeletal pain (McBeth et al., 2010). Accordingly, these findings may have wider applicability to normally cycling females and males as well.

Vitamin D has been touted as an alternative therapy for musculoskeletal pain. Our findings provide a biological basis for advocating dietary supplementation to achieve euvitaminosis D in musculoskeletal pain patients. The controlled experimental conditions directly implicate serum vitamin D deficiency in the etiology of deep muscle pain. Given that vitamin D supplementation is safe, inexpensive, and has few side effects (Heaney, 2008), our findings suggest that it may be a useful prophylactic for muscle pain.

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