Vitamin D_3 Confers Protection from Autoimmune Encephalomyelitis Only in Female Mice[1]

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The prevalence of multiple sclerosis (MS) increases significantly with decreasing UV B light exposure, possibly reflecting a protective effect of vitamin D_3. Consistent with this theory, previous research has shown a strong protective effect 1,25-dihydroxyvitamin D_3 in experimental autoimmune encephalomyelitis (EAE), an MS model. However, it is not known whether the hormone precursor, vitamin D_3, has protective effects in EAE. To address this question, B10.PL mice were fed a diet with or without vitamin D_3, immunized with myelin basic protein, and studied for signs of EAE and for metabolites and transcripts of the vitamin D_3 endocrine system. The intact, vitamin D_3-fed female mice had significantly less clinical, histopathological, and immunological signs of EAE than ovariectomized females or intact or castrated males. Correlating with reduced EAE, the intact, vitamin D_3-fed female mice had significantly more 1,25-dihydroxyvitamin D_3 and fewer CYP24A1 transcripts, encoding the 1,25-dihydroxyvitamin D_3-inactivating enzyme, in the spinal cord than the other groups of mice. Thus, there was an unexpected synergy between vitamin D_3 and ovarian tissue with regard to EAE inhibition. We hypothesize that an ovarian hormone inhibited *CYP24A1* gene expression in the spinal cord, so the locally-produced 1,25-dihydroxyvitamin D_3 accumulated and resolved the inflammation before severe EAE developed. If humans have a similar gender difference in vitamin D_3 metabolism in the CNS, then sunlight deprivation would increase the MS risk more significantly in women than in men, which may contribute to the unexplained higher MS incidence in women than in men. ***The Journal of Immunology,* 2005, 175: 4119–4126.**

Multiple sclerosis (MS)[3] is a chronic, inflammatory, neurodegenerative disease marked by autoreactive T lymphocyte and mononuclear cell infiltration into the CNS, demyelination, oligodendrocyte loss, and axonal degeneration (1). MS often shows a relapsing-remitting course, later developing into chronic progressive disease. For unknown reasons, MS afflicts women twice as often as men. The etiology of MS is uncertain, but available immunologic, genetic, and epidemiologic data suggest that MS is an autoimmune disease that develops in genetically susceptible individuals who are exposed to as-yet undefined environmental risk factors (2, 3). Identifying these environmental factors and elucidating how they increase autoimmune disease risk could guide new strategies to prevent MS.

There is a very strong inverse correlation between MS disease prevalence and UVB exposure (4–9), implying that UVB exposure is strongly protective with respect to MS. Because UVB exposure is required for vitamin D_3 synthesis and vitamin D_3 has important immunoregulatory functions, we proposed that vitamin D_3 may mediate the protective effects of UVB in MS (10–12). Consistent with this hypothesis, MS risk and severity were lowest among individuals who ingested large amounts of fish oil, a rich vitamin D_3 source (13–15), or took vitamin D supplements (16). Also, the periodicity of MS relapses and remissions correlated with seasonal changes in vitamin D_3 supplies from UVB exposure (17). Furthermore, the transcriptional regulatory functions of the hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) are mediated by the nuclear vitamin D receptor (VDR) (18), and genetic epidemiology studies showed that the *VDR b* allele correlated with MS risk in the Japanese (19, 20). Finally, studies showing that 1,25-$(OH)_2D_3$ markedly inhibited induction of the MS model disease, experimental autoimmune encephalomyelitis (EAE) (21–28), and reversed established EAE disease (25, 29) have provided strong evidence in favor of the hypothesis that vitamin D_3 may perform anti-inflammatory and neuroprotective functions in MS. Thus, a robust and diverse body of evidence supports the hypothesis that vitamin D_3 insufficiency may increase the MS risk and, conversely, that adequate vitamin D_3 may decrease this risk.

Although it is well established that 1,25-$(OH)_2D_3$ inhibits EAE, no experiments have examined whether the hormone precursor, vitamin D_3 (30), can inhibit EAE. We addressed this question in the present research. B10.PL mice were fed diets with or without vitamin D_3, immunized with myelin basic protein (MBP) and evaluated for EAE disease. Surprisingly, we found that vitamin D_3 significantly inhibited EAE in female but not male mice, and ovariectomy (OVX) abrogated this protective effect. Gender-based differences in vitamin D_3 metabolism in the CNS correlated with reduced EAE susceptibility in intact, vitamin D_3-fed female mice. Thus, there was synergy between ovarian tissue and vitamin D_3 with respect to EAE inhibition, with the ovarian tissue controlling vitamin D_3 metabolism and anti-inflammatory functions in the CNS. We discuss a possible mechanism for ovarian control of vitamin D_3 metabolism in the inflamed CNS, the implication that sunlight deprivation may contribute to the female predominance of MS, and the possible combined use of female hormones and vitamin D_3 to reduce the MS risk and/or severity.

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[3] Abbreviations used in this paper: MS, multiple sclerosis; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24-OHase, 1,25-dihydroxyvitamin D_3 24-hydroxylase; 25-$(OH)D_3$, 25-hydroxyvitamin D_3; −D, mice fed no vitamin D_3; +D, mice fed 1 μg/day of vitamin D_3; EAE, experimental autoimmune encephalomyelitis; MBP, myelin basic protein; OVX, ovariectomized; SHAM, sham-operated; VDR, vitamin D receptor.

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Materials and Methods

Mice

The B10.PL(73NS)/Sn mice were obtained originally from The Jackson Laboratory and bred in the pathogen-free mouse colony of the Department of Biochemistry. Mice were housed at 25°C with a 12-h light-dark cycle and 40–60% humidity. The drinking water was provided ad libitum. Before experiments, the mice were fed commercial mouse chow containing 0.33 μg/day vitamin D_3 and 1% calcium (lab diet no. 5008; PMI Nutrition International). All animal experimentation was conducted in accord with accepted standards of humane animal care, as outlined in the ethical guidelines. The Institutional Animal Care and Use Committee approved the experimental protocols.

Experimental diets

The synthetic diet was formulated to contain all essential nutrients, except vitamin D_3 (31). The vitamin D_3 (cholecalciferol; Acros Organics) was dissolved in absolute ethanol (1 mg/ml) and stored in the cold. Each 1 kg of synthetic diet was suspended in 1.5 liters of boiling 1.0% agar (Difco) in water and cooled. Before the mixture cooled completely, an amount of vitamin D_3 was added to the diet to provide 0 (−D diet), 1 (+D diet), or 5 μg/day vitamin D_3, calculated based on a daily measured consumption of 4.0 g dry weight of diet/mouse. An oil-soluble vitamin mix without vitamin D_3 was then added to the cooled diet. Fresh synthetic diet was prepared weekly, stored at 4°C, and provided to the mice three times per week.

EAE induction and evaluation

Age- and sex-matched groups of mice were continuously fed the +D or −D diet beginning at age 4 wk. EAE was induced at age 8 wk by injecting MBP isolated from guinea pig spinal cords and pertussis toxin (List Biological Laboratories) as described previously (25). EAE disease severity was evaluated daily as follows: 0, normal; 1, limp tail; 2, paraparesis with a clumsy gait; 3, hind limb paralysis; 4, hind- and fore-limb paralysis; and 5, moribund. At several times during the study, mice were weighed, and blood samples were obtained from the tail vein. At the conclusion of the study, the mice were euthanized, a blood sample was obtained, perfusion was done, and the spinal cords were removed as described previously (25). The spinal cords were flash frozen with liquid nitrogen and stored at −70°C before RNA or 1,25-$(OH)_2D_3$ extraction. Alternatively, the spinal cords were divided into six equal sections, aligned vertically, snap frozen in OCT compound (Sakura Finetek) and stored at −70°C for histopathology. The blood was clotted and centrifuged, and the decanted serum was frozen at −70°C before analysis.

Histopathology

The frozen spinal cords, divided into six equal sections to ensure a representative view of the spinal cord, were cryosectioned transversely at 10 μm, fixed in 37% paraformaldehyde, stained with Gill's No. 3 H&E Y (Sigma-Aldrich), and examined using a Zeiss Axioskop microscope equipped with a Plan-Neofluar ×20/0.5 objective. Bright field images were acquired with AxioVision 3.0 software controlling an Axiocam digital camera. Each spinal cord section was divided into quadrants; 20 quadrants/slide and 2 slides/mouse were scored. The meninges, gray matter, and white matter of the 40 quadrants were scored in a blinded fashion as 0, 1, 2 or 3, based on the presence or the absence of infiltrating cells in each of the regions on the spinal cord. The histopathology score was recorded as the percentage of spinal cord quadrants that showed a readily identifiable inflammatory cell infiltrate.

25-(OH)D and 1,25-$(OH)_2D_3$ analysis

The serum was extracted, and the 25-hydroxyvitamin D_3 (25-$(OH)D_3$) (DiaSorin) and 1,25-$(OH)_2D_3$ (Nichols Institute) concentrations were determined in duplicate with radioimmunoassay kits, according to the manufacturer's protocols. The spinal cords were first extracted with a chloroform-methanol-4% KCl in water (1:2:0.8 v/v) mixture to recover the vitamin D metabolites (32). The spinal cord extracts were then assayed in duplicate for 1,25-$(OH)_2D_3$. A spike-recovery control was performed with each 1,25-$(OH)_2D_3$ extraction by adding 100 pg of 1,25-$(OH)_2D_3$ to a crushed spinal cord from a vitamin D_3-depleted mouse (fed −D diet for >28 days). The extraction was then completed, the 1,25-$(OH)_2D_3$ was assayed in duplicate, the percentage recovery was calculated, and a recovery correction factor was applied to the experimental data. The hormone recovery averaged 70 ± 10%.

Spinal cord transcript analysis

The GAPDH, IFN-γ, VDR, CYP27B1, and CYP24A1 transcript abundance was measured in the spinal cord by real-time PCR. Total cellular RNA was extracted from frozen spinal cord samples using TRI Reagent (Molecular Research Center), and 5 μg were reversed transcribed from an oligo(dT) primer using the Reverse Transcription System (Promega). To assess whether the RNA was intact, the GAPDH transcripts were PCR amplified. Only RNA samples that yielded a GAPDH amplicon were used for further study.

Real-time PCR was performed as described with minor modifications (33). The PCR (25 μl) contained 0.5–1 μg of cDNA, 50 nM of each primer, 12.5 μl of 2× SYBRGreen PCR Master Mix (Applied Biosystems), and 2.25 μl of H_2O. The amplification was accomplished with a GeneAmp 5700 Sequence Detection Systems instrument (Applied Biosystems) programmed for incubations of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 1 min at 60°C. Published primers for the GAPDH, IFN-γ, and CYP27B1 were used (34, 35). The VDR primers were designed using Primer Express Software (Applied Biosystems); they were forward, 5′-GCAACAGCACATTATCGCCAT-3′, and reverse, 5′-TACGTCTGCAC GAATTGGAGG-3′. The CYP24A1 primers, designed by Dr. J. W. Pike (Department of Biochemistry, University of Wisconsin, Madison, WI) were forward, 5′-ACCCCCAAGGTCCGTGACATC-3′, and reverse, 5′-CCAGTTGGTGGGTCCAGGTAAGG-3′. Primers were purchased from Integrated DNA Technologies or Invitrogen Life Technologies. To generate a standard curve, cDNA representing each specific amplicon was gel purified and quantified by absorbance at 260 nm. Each real-time PCR included reactions with serially-diluted standard cDNA. The standard cDNA copy number, calculated from the absorbance and the dilution, was plotted vs the threshold cycle, C_T. The transcript copy number in each unknown sample was determined from C_T by reference to the appropriate standard curve. The data were calculated as transcript abundance relative to GAPDH as the internal standard.

Data analysis

Individual mice were analyzed, and the mean and SD or SEM were calculated for each group of mice. The group sizes are given in the table and figure legends. The significance of differences between the group means was determined using the Mann-Whitney rank sum test, Student's *t* test, Block analysis, or χ^2 test as indicated (36). A value of $p < 0.05$ was considered significant.

Results

The experiments reported here used the EAE model to investigate how vitamin D_3 nutrition might influence MS risk. Mice were fed synthetic diets that provided 0 (−D diet) or 1 μg/day (+D diet) of vitamin D_3 continuously beginning at age 4 wk. The +D diet had three times as much vitamin D_3 as the commercial laboratory mouse diet. At age 8 wk, the mice were primed with MBP and subsequently evaluated for EAE disease. The +D female mice had a significantly lower EAE incidence, peak severity, and cumulative disease index than the −D females or the +D or −D males; none of which differed significantly for any EAE disease parameter (Fig. 1*A*; Table I) . Spinal cord IFN-γ transcript analysis and histopathology corroborated the clinical data. The +D female spinal cord had few IFN-γ transcripts (1 ± 1 copies/10^3 GAPDH copies; $p < 0.02$) 10 days postpriming, when IFN-γ synthesis peaks, whereas the −D female and +D and −D male spinal cord had many IFN-γ transcripts (5–23 copies/10^3 GAPDH copies). Furthermore, the +D female mice showed very little pathology on day 22, the first plateau of acute clinical disease, whereas the −D females and +D and −D males had lesions with inflammatory cell infiltration (Fig. 1*B*). Taken together, the clinical, histological, and immunological data show that vitamin D_3 decreased EAE disease in female but not male mice.

A second study tested a higher vitamin D_3 dose in male mice to determine whether males simply required more vitamin D_3 than females for vitamin D_3-mediated protection from EAE. Feeding 5 μg/day vitamin D_3 elevated the serum 25-$(OH)D_3$ to 133 ± 43 nmol/L, increased the serum calcium, and decreased the body

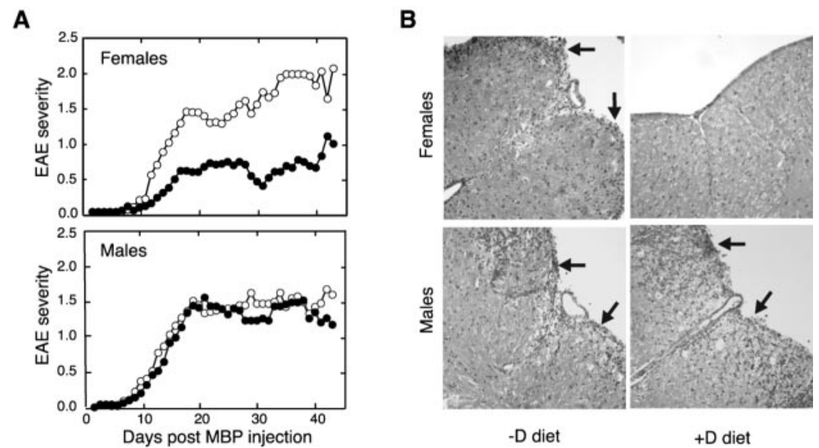

FIGURE 1. Vitamin D_3 inhibited EAE in female but not male mice. *A*, Clinical EAE disease in +D and −D mice. Adult mice were fed synthetic diets formulated to provide 0 (○) or 1 μg/day (●) of vitamin D_3 for 30 days. EAE was induced, synthetic diet feeding was continued, and disease severity was scored daily. Shown is the composite mean from 25 to 28 mice/group evaluated in four separate experiments. The female groups were significantly different on days 11–21 and day 26 onward, whereas the +D female and male groups were significantly different on days 10–14, 16–23, and 25–38 ($p < 0.05$; Student's *t* test). *B*, Histopathological EAE disease in +D and −D mice (representative images from three mice per group). Spinal cords were collected 22 days post-MBP immunization, snap frozen, cryosectioned, fixed, and stained. The arrows highlight inflammatory lesions.

weight, indicating these mice had vitamin D_3 toxicity. However, this higher vitamin D_3 dose still did not significantly reduce any EAE disease parameter in male mice (data not shown). Therefore, increasing the vitamin D_3 dose 5-fold did not protect males from severe EAE.

Seeking an explanation for the sex-based disparity in EAE susceptibility in mice fed a high vitamin D_3 diet, we analyzed vitamin D_3 metabolism. As expected, the serum 25-(OH)D_3 increased in the +D female and male mice and decreased in the −D mice during 1 mo of synthetic diet feeding before immunization (Fig. 2*A*). At the time of immunization, there were no gender differences in serum 25-(OH)D_3. In the first 10 days after EAE induction, the +D females continued to increase their serum 25-(OH)D_3, whereas the +D males did not. Consequently, from day 10 post-MBP priming onward, the serum 25-(OH)D_3 was significantly higher in the +D females than in the +D males. Thus, EAE susceptibility correlated inversely with a sex-based disparity in serum 25-(OH)D_3.

In contrast to the serum 25-(OH)D_3, which varied with gender, diet, and EAE induction, the serum 1,25-$(OH)_2D_3$ did not vary significantly with gender, diet, or EAE induction. The unprimed +D mice (males, 159 ± 47 pmol/L; females, 133 ± 79 pmol/L) and −D mice (males, 169 ± 77 pmol/L; females, 145 ± 50 pmol/L) had equivalent serum 1,25-$(OH)_2D_3$ after 1 mo of synthetic diet feeding. At the termination of the experiment, the serum 1,25-$(OH)_2D_3$ values were not significantly different from the unprimed values and continued to show no variation with gender or diet (Fig. 2*B*). Thus, EAE susceptibility did not correlate with serum 1,25-$(OH)_2D_3$.

The spinal cord 1,25-$(OH)_2D_3$ is most relevant to disease outcome, because activated macrophages would be expected to produce 1,25-$(OH)_2D_3$ in the CNS, and EAE pathology is confined to the CNS. However, the 1,25-$(OH)_2D_3$ in the CNS has not been determined and reported for humans or rodents under any conditions. We developed a method to extract and quantify 1,25-$(OH)_2D_3$ from spinal cords with the aid of spike-recovery controls. Before priming, the spinal cord 1,25-$(OH)_2D_3$ was 30–44 fmol/g and did not vary with gender or diet. At the termination of the experiment, it was significantly higher in +D females than in −D females or +D or −D males (Fig. 2*C*). Thus, EAE susceptibility correlated inversely with a sex-based disparity in spinal cord 1,25-$(OH)_2D_3$. Importantly, the serum and spinal cord 1,25-$(OH)_2D_3$ levels did not correlate, suggesting that some spinal cord 1,25-$(OH)_2D_3$ was produced in situ. To our knowledge, this is the first direct evidence for synthesis of 1,25-$(OH)_2D_3$ in the CNS.

The higher spinal cord 1,25-$(OH)_2D_3$ in the MBP-primed +D females might be due to increased hormone synthesis or decreased hormone inactivation. Hormone synthesis and inactivation rates are proportional to the CYP27B1 and CYP24A1 transcripts (37). The *CYP27B1* gene encodes the rate-limiting 25-dihydroxyvitamin D_3-1-α-hydroxylase for hormone synthesis, whereas the *CYP24A1* gene encodes the 1,25-dihydroxyvitamin D_3 24-hydroxylase (24-OHase) that degrades 1,25-$(OH)_2D_3$ (37*)*. Therefore, to evaluate 1,25-$(OH)_2D_3$ synthesis and inactivation rates, the spinal cord

Table I. *Dietary vitamin D_3 delayed the onset and reduced the severity of EAE in female but not male mice*[a]

Sex	Dietary Vitamin D_3 (μg/day)	Incidence[b] (%)	Onset (day)	Mortality (%)	Peak Disease Severity	Cumulative Disease Index[c]	Sections with Lesions (%)
Female	0	100	16 ± 5	18	2.7 ± 1.2	45 ± 35	63 ± 15
Female	1	68**##	19 ± 7	0*	1.3 ± 0.7**##	18 ± 15**#	25 ± 17**##
Male	0	100	16 ± 6	4	2.3 ± 1.0	33 ± 21	67 ± 8
Male	1	100	19 ± 10	0	2.0 ± 0.8	32 ± 19	65 ± 3
Female SHAM[d]	1	73††	16 ± 5††	31	1.7 ± 1.3	9 ± 9†	
Female OVX[d]	1	100	12 ± 4	11	2.3 ± 1.0	16 ± 9	

[a] The experimental design is presented in the Fig. 1 legend. The clinical data shown are the composite mean ± SD from four separate experiments (15–28 mice/group). The histopathology data are from three mice per group. The statistical tests performed on the data were the χ^2 test (incidence and mortality), the Student *t* test (disease onset, severity, and cumulative disease index), and the Mann-Whitney rank sum test (histopathology data). For within-sex comparisons, the * indicates $p < 0.05$ and ** indicates $p < 0.01$. For between-sex comparisons, the # indicates $p < 0.05$ and ## indicates $p < 0.01$. For SHAM and OVX group comparisons, the † indicates $p < 0.05$ and †† indicates $p < 0.01$.

[b] Mice with a clinical score ≥ 1 for 2 consecutive days were considered to have EAE.

[c] The cumulative disease index is the sum of the clinical scores for the 42 days postimmunization for the original experiments and 22 days postimmunization for the surgery experiments.

[d] After 30 days of the synthetic diet feeding, the female mice were SHAM or OVX, allowed to recover from surgery for 10 days, and primed with MBP while diet feeding continued throughout the study.

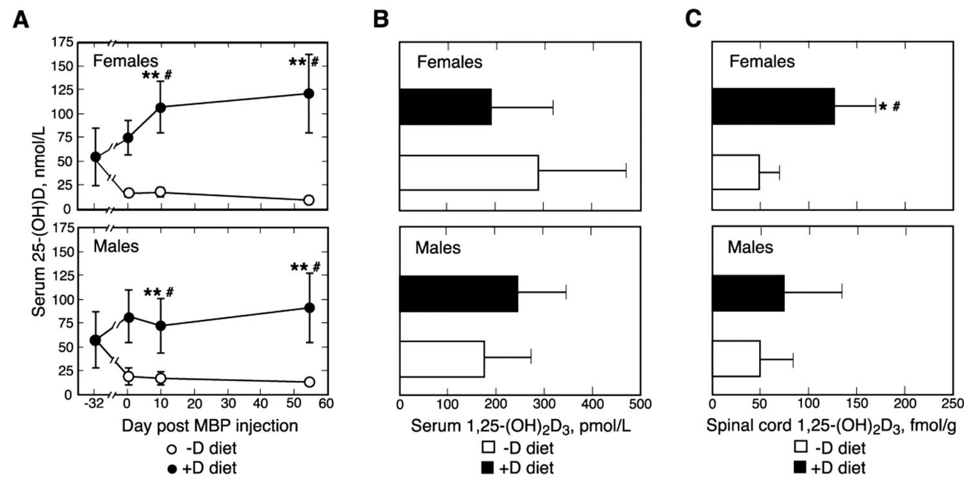

FIGURE 2. The serum 25-$(OH)D_3$ and spinal cord 1,25-$(OH)_2D_3$ levels were higher in the MBP-primed +D female mice than in their male counterparts. *A*, Serum 25-$(OH)D_3$ in MBP-primed +D and −D mice. The experiment was performed as detailed in the Fig. 1 legend. Shown is the composite mean ± SD for 10–17 mice/group. For within-sex comparisons, ** indicates $p < 0.001$; for between-sex comparisons, # indicates $p < 0.05$ (Student's *t* test). *B*, Serum 1,25-$(OH)_2D_3$ in MBP-primed +D and −D mice. Shown is the composite mean ± SD for samples collected 56 days postpriming (six to eight mice per group). *C*, Spinal cord 1,25-$(OH)_2D_3$ in MBP-primed +D and −D mice. Shown is the mean ± SD (corrected for hormone recovery) from one of four experiments (8–12 samples/group). Samples were collected 42 or 56 days post-MBP priming. For within-sex comparisons, * indicates $p < 0.01$; for between-sex comparisons, # indicates $p < 0.01$ (Block analysis).

CYP27B1 and CYP24A1 transcripts were measured. These transcripts were analyzed on day 10 post-MBP priming, when clinical signs attributable to activated macrophages in the CNS were first evident. We reasoned that differences in synthesis or inactivation early in the disease process would have the greatest impact on disease outcome. The kidney CYP27B1 and CYP24A1 transcripts were also quantified. The data showed that the kidney and spinal cord CYP27B1 transcripts (Table II) and the kidney CYP24A1 transcripts (Fig. 3*A*) did not vary significantly with gender, diet, or MBP priming. In sharp contrast, the spinal cord CYP24A1 transcripts varied significantly with gender and MBP priming (Fig. 3*B*). All mice had similar CYP24A1 transcript levels in the CNS before priming, and the CYP24A1 transcripts decreased after priming. However, the MBP-primed females prevented CYP24A1 gene transcription more completely than the MBP-primed males. The +D females also had a higher CYP27B1:CYP24A1 transcript ratio than the +D males (17 ± 2 compared with 6 ± 2; $p < 0.001$). Thus, EAE susceptibility correlated directly with a sex-based disparity in spinal cord CYP24A1 transcripts and inversely with the spinal cord 1,25-$(OH)_2D_3$. One interpretation of these data is that +D females and males produced spinal cord 1,25-$(OH)_2D_3$ at similar rates, but the +D females inactivated it more slowly, allowing it to accumulate and inhibit EAE. The interpretation that males inactivated 1,25-$(OH)_2D_3$ more rapidly could explain why substantially more 1,25-$(OH)_2D_3$ was needed to prevent EAE in males than in females (24).

The decreased spinal cord CYP24A1 mRNA in the MBP-primed +D females might be due to decreased VDR expression because the multiple vitamin D-responsive elements in the *CYP24A1* promoter couple gene expression to VDR activity (38). Normal mice had one to three VDR copies per 10^3 GAPDH copies in the spinal cord, regardless of gender or diet (Table II). These transcripts increased 10 days post-MBP priming with the increase in inflammatory cell infiltration into the CNS but still did not vary significantly with gender or diet. The +D males had a substantially higher CYP24A1:VDR transcript ratio than the +D females

Table II. *The spinal cord CYP24A1 transcripts decreased with MBP priming to a significantly lower level in the female mice than in the male mice*[a]

			Transcript Copies/10^3GAPDH Copies by Sex and Dietary Vitamin D_3			
			Female		Male	
Transcript	Sample	Day Relative to MBP Priming	−D	+D	−D	+D
VDR	CNS	0	2.3 ± 0.6	2.2 ± 0.7	0.7 ± 0.2	3.0 ± 1.0
VDR	CNS	10	25.7 ± 7.0	18 ± 5.4	50.2 ± 14.2	14.2 ± 5.0
CYP27B1	Kidney	0	0.9 ± 0.2	4.8 ± 2.2	2.2 ± 0.7	7.4 ± 2.2
CYP27B1	Kidney	22	2.0 ± 1.0	1.6 ± 1.2	4.9 ± 3.5	0.9 ± 0.5
CYP27B1	CNS	0	3.4 ± 1.2	3.3 ± 1.2	3.0 ± 1.5	4.4 ± 1.3
CYP27B1	CNS	10	3.9 ± 1.0	4.2 ± 1.1	19.6 ± 6.1	10.2 ± 3.2
CYP24A1	Kidney	0	1.3 ± 0.3	2.9 ± 1.4	1.9 ± 1.0	5.8 ± 1.9
CYP24A1	Kidney	22	1.7 ± 0.6	1.6 ± 0.4	1.2 ± 0.3	2.2 ± 0.8
CYP24A1	CNS	0	2.7 ± 1.4	2.1 ± 0.8	2.9 ± 1.8	7.8 ± 3.5
CYP24A1	CNS	10	0.4 ± 0.1	0.4 ± 0.1[#]	2.2 ± 0.8	1.6 ± 0.4[#]

[a] The experimental design is presented in Fig. 1 legend. The samples were collected on the indicated days; CNS designates spinal cord samples. The RNA was extracted, and a real-time PCR analysis was performed in triplicate. The values shown are the composite mean ± SEM for three separate experiments. The day 0 and day 22 data are from 5–7 samples/group. The day 10 data are from 8–14 samples/group. For between-gender comparisons, # indicates $p < 0.01$ (Mann-Whitney rank sum test).

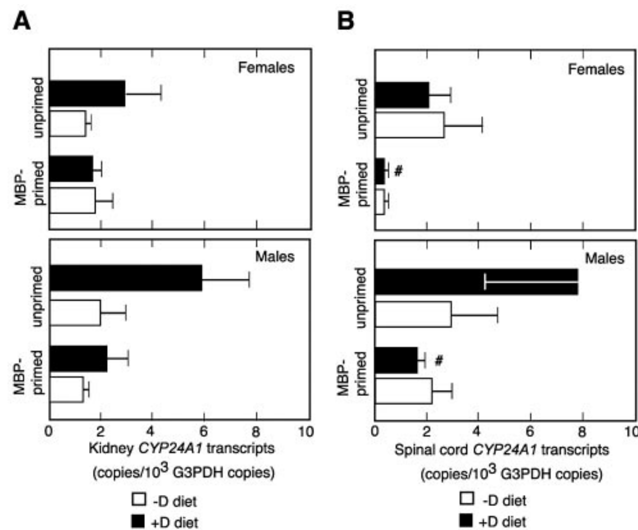

FIGURE 3. The spinal cord CYP24A1 transcript level was lower in the +D female mice immunized with MBP than in their male counterparts. *A*, Kidney CYP24A1 transcripts in +D and −D mice immunized with MBP. The experiment was performed as detailed in the Fig. 1 legend. Shown is the composite mean ± SEM for five to seven samples per group collected 22 days post-MBP priming and analyzed in triplicate by real-time PCR. *B*, Spinal cord CYP24A1 transcripts in +D and −D mice immunized with MBP. Shown is the composite mean ± SEM for 6–7 nonimmune mice/group or 8–12 MBP-primed mice/group (10 days post-MBP priming) analyzed in triplicate by real-time PCR. For between-sex comparisons, # indicates $p < 0.01$ (Mann-Whitney *U* test).

(0.27 ± 0.12 compared with 0.02 ± 0.01; $p < 0.01$), supporting the interpretation that the sex-based difference in CYP24A1 transcripts was due to a VDR-independent difference in *CYP24A1* gene induction.

At least two mechanisms might explain why the MBP-primed +D females prevented *CYP24A1* gene induction more completely than the +D males. Female hormones might directly or indirectly repress this gene or male hormones might stimulate gene expression. To distinguish these possibilities, mice were continuously fed a +D or −D diet beginning 30 days before OVX, castration, or sham surgery (SHAM). Ten days postsurgery, they were primed with MBP, diet feeding was continued, and EAE disease was analyzed. The castrated, MBP-primed +D and −D males did not differ significantly for any EAE disease parameter, indicating that male hormones did not impede vitamin D_3-mediated disease prevention (data not shown). The SHAM +D females had a delayed onset and less severe EAE than the SHAM −D females, confirming that vitamin D_3 protected intact female mice (Fig. 4*A*). However, vitamin D_3 did not protect the OVX, MBP-primed +D females (Fig. 4*A*), and these females did not accumulate 1,25-$(OH)_2D_3$ in the spinal cord (Fig. 4*B*). These results support the interpretation that male hormones did not suppress 1,25-$(OH)_2D_3$ accumulation in the spinal cord, rather, female hormones directly or indirectly facilitated this accumulation.

Discussion

We have provided evidence that nontoxic levels of dietary vitamin D_3 inhibited severe EAE only in female mice. This striking sex-based disparity in EAE susceptibility apparently derives from sex-based differences in vitamin D_3 metabolism in the CNS. The two sexes of mice had equivalent serum 1,25-$(OH)_2D_3$ concentrations, but in the CNS, the relatively EAE disease-free +D females had significantly less CYP24A1 mRNA encoding the inactivating 24-OHase enzyme and therefore higher 1,25-$(OH)_2D_3$ levels than the EAE-diseased +D males. The higher 1,25-$(OH)_2D_3$ level provided very potent anti-inflammatory and neuroprotective activities in the intact +D females. OVX eliminated this protective mechanism. The OVX female mice did not accumulate 1,25-$(OH)_2D_3$ in the CNS. Lacking the 1,25-$(OH)_2D_3$-mediated anti-inflammatory and neuroprotective functions, the OVX females sustained severe inflammation-induced CNS damage and progressive motor function loss. Our data provide the first direct evidence that the ovary regulates vitamin D_3 metabolism and 1,25-$(OH)_2D_3$-mediated anti-inflammatory and neuroprotective functions in the CNS.

Precisely how 1,25-$(OH)_2D_3$ inhibits EAE is still not entirely clear (12). Many in vitro experiments have shown that 1,25-$(OH)_2D_3$ prevented immature dendritic cells from maturing and producing the costimulatory molecules and cytokines needed for priming T lymphocytes (39), but we found no evidence for this mechanism in vivo in the EAE model system (27). Other in vitro experiments have suggested that 1,25-$(OH)_2D_3$ may facilitate Th type 2 responses rather than Th type 1 responses, but we and others (12, 26, 27) found no evidence for this type of activity in vivo in the EAE model system. We found a requirement for *Rag-1*-dependent cells other than the encephalitogenic Th type 1 cells in the mechanism (27), whereas others found that $CD8^+$ T cells were not essential (28). We interpreted these data as suggesting that the 1,25-$(OH)_2D_3$ may be supporting the function of regulatory T cells that maintain peripheral tolerance to self (27). Other data from our lab showed that 1,25-$(OH)_2D_3$ reduced the number of inflammatory cells in the CNS by sensitizing these cells to apoptotic signals (25, 29). Additional experiments are underway to define more precisely how 1,25-$(OH)_2D_3$ inhibits EAE.

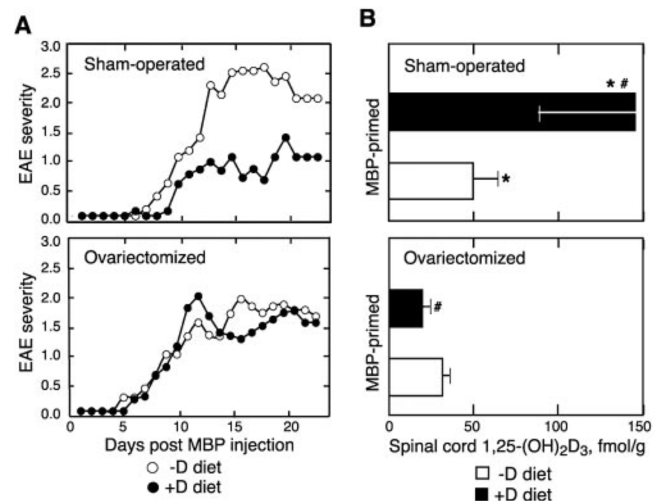

FIGURE 4. The +D OVX female mice had low spinal cord 1,25-$(OH)_2D_3$ levels and developed EAE after MBP priming. *A*, Clinical EAE disease in +D and −D, SHAM, and OVX female mice. Adult female mice were fed +D or −D diet throughout the study. After 30 days of diet feeding, they were OVX or SHAM. Ten days after surgery, they were immunized to induce EAE. Disease severity was recorded daily after MBP priming. Shown is the composite mean for 13–15 mice/group evaluated in three separate experiments. The +D and −D SHAM females were significantly different on days 15–18 ($p < 0.05$; Mann-Whitney *U* test). *B*, Spinal cord 1,25-$(OH)_2D_3$ in +D and −D OVX and SHAM female mice immunized with MBP. Shown is the mean ± SD (corrected for hormone recovery) from one of two experiments (five to nine samples per group). Samples were collected 22 days post-MBP priming. The +D SHAM females were significantly different from the −D SHAM females (*, $p < 0.05$; Block analysis) and from the +D OVX females (#, $p < 0.05$; Block analysis).

The nuclear VDR is expressed in the CNS (40, 41), and the 1,25-$(OH)_2D_3$ performs a variety of neuroprotective functions that reduce CNS damage and motor function loss. The VDR was detected in the hippocampus (neurons and astrocytes), where it appeared to support hippocampal cell survival (42), and in brain regions involved in memory and cognition, implicating 1,25-$(OH)_2D_3$ in memory processing (43). The VDR was also present in hypothalamic neurons, implicating 1,25-$(OH)_2D_3$ in the control of hypothalamic peptidergic systems (44), and in oligodendrocytes (45) and astrocytes (46), suggesting possible roles for 1,25-$(OH)_2D_3$ in the control of myelination and blood-brain barrier maintenance. We found that 1,25-$(OH)_2D_3$ regulated the cadherin-related neuronal receptor I, glial fibrillary acidic protein, and other genes associated with neuroprotection (29). The 1,25-$(OH)_2D_3$ stimulated the synthesis of neurotrophins, including nerve growth factor (46, 47), a neurotrophin that performs differentiative, protective, and repair functions with respect to sensory and sympathetic neurons (48). It also stimulated the synthesis of neurotrophic tyrosine kinase receptor type 2 (29) and other neurotrophin receptors (49). Furthermore, the 1,25-$(OH)_2D_3$ enhanced the expression of Ca^{2+} binding proteins (29, 50) and decreased the mRNAs encoding the α(1C) and α(1D) pore-forming subunits of the L-type Ca^{2+} channels (51). These 1,25-$(OH)_2D_3$-mediated changes would be expected to reduce Ca^{2+} and decrease the vulnerability of neurons to excitotoxic damage, because Ca^{2+} channel increases enhanced neuronal sensitivity to excitotoxic insults and correlated with brain aging and motor impairments.

Sunlight exposure provides >90% of the human vitamin D_3 requirement (30). In our experiments, we varied the dietary vitamin D_3 levels in the +D and −D mice to model humans with high and low sunlight exposure, respectively. In humans, the serum 25-$(OH)D_3$ varies with sunlight exposure and dietary vitamin D_3 (52), although the serum 1,25-$(OH)_2D_3$ does not (53). People with plentiful sunlight exposure, e.g., individuals living at low latitudes and working outdoors (lifeguards or farmers), had ~100–200 nmol/L serum 25-$(OH)D_3$ (52). Conversely, sunlight-deprived individuals had ~10–40 nmol/L serum 25-$(OH)D_3$ (54); examples are people living at latitudes >34°N or °S, where vitamin D_3 synthesis does not occur year-round (55) or living and working mainly indoors (submarine sailors, office workers, the homebound elderly, children in school and daycare). By comparison, the +D mice had 80–120 nmol/L serum 25-$(OH)D_3$, whereas the −D mice had 5–20 nmol/L, similar to humans with high or low sunlight exposure, respectively. To sustain >70 nmol/L serum 25-$(OH)D_3$ during the winter months, healthy men required an estimated 3000–4000 IU of vitamin D_3 daily (52, 54, 56, 57).

To explain why vitamin D_3 did not protect OVX female mice, we hypothesize that estrogen derived from ovarian tissue was essential to prevent *CYP24A1* gene induction and allow the 1,25-$(OH)_2D_3$ to accumulate and mediate anti-inflammatory and neuroprotective functions. This hypothesis is based on data showing that estrogen is protective in MS and EAE. In MS, relapse rates declined during pregnancy and escalated postpartum, concurrently with pregnancy-induced changes in estrogen levels (58, 59). Moreover, estrogen therapy reduced the MS severity in nonpregnant, female MS patients (60, 61). A small study found that most MS patients experienced more severe symptoms with declining estrogen levels after menopause (62). With respect to EAE, female mice were more susceptible to EAE than males (63), and OVX females had accelerated EAE disease (64). Pregnancy protected female mice from EAE induction and progression (64–67). Moreover, estrogen treatment of normal or OVX females at the time of EAE induction suppressed EAE disease (64, 68, 69), whereas progesterone treatment had no effect (70). It is possible that the protective functions of estrogen in MS and EAE may reflect in part a role for estrogen in regulating vitamin D_3 metabolism in the CNS. Ongoing studies are testing this hypothesis by evaluating vitamin D_3-medated inhibition of EAE in OVX mice with and without hormone replacement.

There is substantial published evidence that estrogen stimulates 1,25-$(OH)_2D_3$ accumulation in women as we found in female mice. Firstly, the serum 1,25-$(OH)_2D_3$ increased 2-fold from the early follicular phase of the menstrual cycle, when estrogen levels are low, to the time of ovulation, when estrogen levels are high (71, 72). Secondly, the serum 1,25-$(OH)_2D_3$ rose 2- to 3-fold during the high estrogen period of pregnancy (73). Finally, the serum 1,25-$(OH)_2D_3$ increased >50% in young women (74) and postmenopausal women (75–78) receiving estrogen therapy. These data establish a cause-effect relationship between increased estrogen and higher 1,25-$(OH)_2D_3$ levels in the serum. Moreover, the data are consistent with the hypothesis that estrogen may strongly influence vitamin D_3 metabolism and the anti-inflammatory and neuroprotective functions of the vitamin D_3 endocrine system in the CNS. In this way estrogen, sunlight, and vitamin D_3 may function synergistically to reduce MS risk and/or severity in women.

It is not known why female and male mice had equivalent *CYP24A1* gene expression in the CNS before MBP priming, but the intact, female mice had significantly lower *CYP24A1* gene expression than males after MBP priming. In the kidney, there is a feedback inhibition loop wherein the 1,25-$(OH)_2D_3$ and the VDR strongly induce the *CYP24A1* gene encoding the hormone-inactivating 24-OHase through dual vitamin D-responsive elements in the *CYP24A1* gene promoter (38). The 24-OHase then converts 1,25-$(OH)_2D_3$ into an inactive metabolite, thereby maintaining the plasma 1,25-$(OH)_2D_3$ concentration within very narrow limits (37). Under noninflammatory conditions in the CNS, the 1,25-$(OH)_2D_3$ concentration may also be maintained within narrow limits because glial cells express the VDR and reportedly induce the *CYP24A1* gene in response to 1,25-$(OH)_2D_3$ (79). However, the feedback inhibition loop does not operate in activated macrophages, where IFN-γ signaling prevents *CYP24A1* gene induction and allows the 1,25-$(OH)_2D_3$ to accumulate (80).

We theorize that female mice had lower *CYP24A1* gene expression in the CNS than males after MBP priming because of estrogen-related differences in IFN-γ synthesis and signaling in activated macrophages. Estrogen reportedly stimulated IFN-γ production via an estrogen-responsive element in the IFN-γ promoter (81). Moreover, IFN-γ signaling activated Stat1, and activated Stat1 prevented the VDR from transactivating the *CYP24A1* promoter (82). Thus, the intact females, through estrogen action, may have produced more IFN-γ in the CNS soon after MBP priming, and the IFN-γ may have rapidly and completely terminated *CYP24A1* gene expression in the activated macrophages, allowing the 1,25-$(OH)_2D_3$ to accumulate in the spinal cord. By day 10, when clinical disease signs were first evident in the males and the −D females, the +D females may have partially resolved the inflammation and decreased the IFN-γ production, explaining why the IFN-γ transcripts were not abundant in this group at this time point. This interpretation is consistent with an unexplained protective role of IFN-γ in EAE (83, 84). Current experiments are testing this hypothesis through studies of vitamin D_3-mediated inhibition of EAE in intact and OVX female mice with a targeted disruption of the *IFN-γ* gene.

The research reported here has significant implications for MS. If humans have a similar gender difference in vitamin D_3 metabolism in the CNS, then sunlight deprivation would increase the MS risk more significantly in women than in men. In this manner, a

gender bias with regard to benefits from a protective environmental factor could introduce a gender bias in disease incidence, relating to the unexplained female bias in MS incidence (85). If men do not completely prevent *CYP24A1* gene induction in activated macrophages, then they may not benefit from sunlight's protective effects or from vitamin D_3-based therapeutic strategies to inhibit MS. A second important implication is that the protective effects of sunlight and vitamin D_3 may decline significantly in menopausal women, when there are decreasing supplies of estrogen to prevent *CYP24A1* gene induction and promote the anti-inflammatory and neuroprotective functions of 1,25-$(OH)_2D_3$. This concern raises the possibility of combining hormone replacement therapy and UVB exposure or high dietary vitamin D_3 to slow MS progression or reduce MS severity in peri- and postmenopausal women. It is our hope that these insights will guide new strategies to reduce the prevalence and impact of MS and possibly other autoimmune diseases.

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Disclosures

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