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Estrogen Controls Vitamin D₃-Mediated Resistance to Experimental Autoimmune Encephalomyelitis by Controlling Vitamin D₃ Metabolism and Receptor Expression¹

Faye E. Nashold,* Karen M. Spach,† Justin A. Spanier,* and Colleen E. Hayes²*  

Multiple sclerosis (MS) is an autoimmune, neurodegenerative disease with a rapidly increasing female gender bias. MS prevalence decreases with increasing sunlight exposure, supporting our hypothesis that the sunlight-dependent hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) is a natural inhibitor of autoimmune T cell responses in MS. We found that vitamin D₃ inhibited experimental autoimmune encephalomyelitis (EAE) in intact female mice, but not in ovariectomized females or males. To learn whether 17β-oestradiol (E₂) is essential for vitamin D₃-mediated protection, ovariectomized female mice were given E₂ or placebo and evaluated for vitamin D₃-mediated enhancement of EAE resistance. Diestrus-level E₂ implants alone provided no benefit, but they restored vitamin D₃-mediated resistance in the ovariectomized females. Synergy between E₂ and vitamin D₃ occurred through vitamin D₃-mediated enhancement of E₂ synthesis, as well as E₂-mediated enhancement of vitamin D receptor expression in the inflamed CNS. In males, E₂ implants did not enable vitamin D₃ to inhibit EAE. The finding that vitamin D₃-mediated protection in EAE is female-specific and E₂-dependent suggests that declining vitamin D₂ supplies due to sun avoidance might be contributing to the rapidly increasing female gender bias in MS. Moreover, declining E₂ synthesis and vitamin D₃-mediated protection with increasing age might be contributing to MS disease progression in older women.  

The vitamin D endocrine system probably mediates the protective effects of sunlight exposure in MS (9). UV light is required for an early step in the biosynthesis of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃), a secosteroid hormone (10). The unique dependence of 1,25-(OH)₂D₃ synthesis on UV light, and the discovery of vitamin D receptors (VDR) in activated T lymphocytes (11, 12), led us to propose that sunlight’s protective effects in MS might reflect the need for 1,25-(OH)₂D₃ to control the T lymphocyte-mediated autoimmune responses that are pathogenic in MS (9, 13). Since we first proposed this hypothesis, a diverse and compelling body of supporting evidence has emerged from human studies (14). For example, high levels of serum 25-hydroxyvitamin D₃ (25-(OH)D₃), which is biologically inactive, correlated with low MS risk, fewer relapses, and significantly less disability (15–21). Conversely, low 25-(OH)D₃ levels correlated with high MS risk, frequent relapses, and significantly higher disability. In an ongoing vitamin D₃ intervention study, investigators noted a trend toward fewer relapses and stable or improved disability in the supplemented patients compared with the unsupplemented MS patients (22). There is a 3–4 mo lag between the seasonal decreases in UV light exposure and circulating 25-(OH)D₃ levels and the increases in MS relapses (17, 19, 20). This lag suggests that seasonal fluctuations in UV light exposure could contribute to the relapsing-remitting MS disease phenotype. Importantly, low circulating levels of biologically active 1,25-(OH)₂D₃ correlated with a progressive disease course (21). Consistent with the possibility that reduced 1,25-(OH)₂D₃ synthesis might be causal in MS progression, a recent report described three patients with vitamin D-dependent rickets type I who all developed MS (23). Vitamin D-dependent rickets type I is a very rare hereditary disease caused by mutation of the Cyp27b1 gene encoding the enzyme that converts inactive 25-(OH)D₃ into 1,25-(OH)₂D₃. The probability of three coincident vitamin D-dependent rickets type I and MS cases by chance alone is ~3.4 × 10⁻⁹, arguing strongly for a cause-effect relationship between Cyp27b1 mutation, reduced 1,25-(OH)₂D₃

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³Abbreviations used in this paper: MS, multiple sclerosis; 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 25-(OH)D₃, 25-hydroxyvitamin D₃; Cyp, threshold PCR cycle; E₂, 17β-oestradiol; EAE, experimental autoimmune encephalomyelitis; ER, estrogen receptor; MBP, myelin basic protein; MOG, myelin oligodendrocyte glycoprotein; OVX, ovariectomized; SHAM, sham-operated; VDR, vitamin D receptor.

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synthesis, and MS disease. Finally, a clinical trial found that MS patients had 80% fewer relapses and no disability progression while they were receiving 1,25-(OH)₂D₃ treatment, compared with their relapse frequency and disability progression rates before and after treatment (24). Taken together, these human studies strongly support our hypothesis that low 25-(OH)D₃ and 1,25-(OH)₂D₃ levels increase MS disease risk, severity, and progression (9).

There is a rapidly increasing female gender bias in MS that is not understood (25–30). This female gender bias involves sex hormones, because it becomes apparent after sexual maturity (31). It is not clear why the female-to-male sex ratio rises with increasing estrogen synthesis in sexually mature females, because estrogen is protective in both human MS and rodent experimental autoimmune encephalomyelitis (EAE) (32–36). In our EAE studies, we found a link between female sex hormones and vitamin D₃. High-level vitamin D₃ supplementation inhibited EAE in intact adult female mice, but not in ovariecetomized (OVX) females or males (37). Thus, vitamin D₃ provided a female-specific and sex hormone-dependent protective effect in EAE. Interestingly, high serum 25-(OH)D₃ levels correlated with a reduced MS risk in women but not in men (38). If vitamin D₃ were to provide a female-specific and sex hormone-dependent protective effect in MS as in EAE, then this protective effect would become evident at sexual maturity, and after puberty, vitamin D₃ insufficiency would increase MS risk in a female-specific manner.

Here, we investigated the female-specific and sex hormone-dependent protective effect of vitamin D₃ in EAE in more detail. To test the hypothesis that 17β-estradiol (E₂) might regulate vitamin D₃ metabolism and/or VDR expression in the CNS, OVX female mice were implanted with E₂ or placebo pellets and evaluated for vitamin D₃-mediated EAE resistance, vitamin D₃ metabolism, and VDR expression in the CNS. Our data show for the first time that E₂ enables vitamin D₃ to decrease EAE risk and severity in female mice through enhancement of VDR transcription and function in the CNS. We propose a model for the functional synergy between the vitamin D and estrogen endocrine systems, and we discuss our model in the context of the relapsing-remitting phenotype, the female gender bias, and vitamin D₃-based strategies to decrease MS risk and disease severity.

Materials and Methods

Mice

The B10.PL-H₂H₂-T18a/H₂H₂-T18a/H₂H₂-T18a/SN (hereafter B10.PL) and C57BL/6 (hereafter B6) mice were obtained from The Jackson Laboratory. Some B10.PL mice were bred in the pathogen-free mouse colony of the Department of Biochemistry from The Jackson Laboratory-derived breeding pairs. Mice were housed at 25°C with a 12 h light-dark cycle and 40–60% humidity. The drinking water was provided ad libidum. Before experiments, the mice were fed commercial mouse chow containing 0.33 μg/day of vitamin D₃ and 1% calcium (Lab Diet no. 5008; PMI Nutrition International). The Institutional Animal Care and Use Committee approved the experimental protocols. All animal experimentation was conducted in accordance with accepted standards of humane animal care.

Experimental diets

The synthetic diet was formulated to contain all essential nutrients except vitamin D₃, exactly as we described (37). The vitamin D₃ (cholecalciferol; Acros Organics) was dissolved in absolute ethanol (1 mg/ml) and stored in the cold. It was added to the synthetic diet in an amount calculated to provide 0 (−D diet) or 1 μg/day (+D diet) of vitamin D₃ based on a measured daily diet consumption of 4.0 g dry weight of diet per mouse (37). Groups of mice (sex-matched; age 6–8 wk) were fed the −D or +D diet continuously beginning on the first day of each study. Fresh synthetic diet was prepared weekly, stored at 4°C, and provided to the mice three times per week.

Ovariectomy and estradiol therapy

OVX or SHAM surgery was performed 1 wk after synthetic diet feeding began. During the surgery, a controlled release pellet containing E₂ (3 mg; 0.1 mg of E₂) or a placebo was implanted dorsally s.c. using a 12-gauge trochar, as described by the manufacturer (Innovative Research of America).

EAE induction and evaluation

EAE was induced 3 wk after synthetic diet feeding began. The myelin basic protein (MBP), isolated from guinea pig spinal cords and dissolved in 0.1 M acetic acid (4 mg/ml), was emulsified with an equal volume of CFA containing heat-killed Mycobacterium tuberculosis H37 Ra (4 mg/ml). Each anesthetized B10.PL mouse was injected s.c. with 0.2 ml of emulsion (600 μg of MBP) in three sites on the hind flanks. Each mouse was injected i.p. with 200 μg of pertussis toxin (List Biological Laboratories) in 0.1 ml of sterile PBS solution on the day of MBP immunization and again 2 days later. EAE was induced in B6 mice using the same protocol, except that a peptide from mouse myelin oligodendrocyte proteoglycan (MOG35-55 sequence MEVGWVRSFSPSRVVHVLYRNGK) replaced MBP, and the emulsion injection volume was 0.1 ml (200 μg of MOG35-55 peptide).

Disease severity assessment

We used a modified EAE severity scale (25–(OH)D₃) (Alpco Diagnostics) and 1,25-(OH)₂D₃ (ImmunoLogic Systems). The spinal cords were extracted previously (37). A spike-recovery control was performed with each spinal cord transcript analysis. The serum E2 was assayed using an enzyme immunoassay kit (DSL 10-4300; Diagnostic Systems Laboratories). Enzyme immunoassay kits were also used to quantify the serum 1,25(OH)₂D₃ (Immunodiagnostic Systems). The spinal cords were extracted and the extracts assayed in duplicate for 1,25(OH)₂D₃ as described previously (37). A spike-recovery control was performed with each spinal cord extraction, the percentage recovery was calculated, and a recovery correction factor was applied to the experimental data.

Spinal cord transcription analysis

The GAPDH, IFN-γ, VDR, Cyp27b1, and Cyp24a1 transcript abundance was measured in the spinal cord by real-time PCR as described (37). The amplification and detection were accomplished with a 7500 Fast real-time PCR system (Applied Biosystems) and SYBR Advantage quantitative PCR premix (Clontech). Published primers for the GAPDH, IFN-γ, and Cyp27b1 were used (42, 43). The VDR primers were forward 5′-GCA ACA GCA CAT TAT CCC CAT 3′ and reverse 5′-TAC GTC TGC ACG CAT TGG AGG 3′. The Cyp24a1 primers were forward 5′-ACC CCC AAG GTC CGT GAC GAC ATC 3′ and reverse 5′-CCA GTG GGT GGG TCC AGG TAA GGG 3′. Primers were purchased from Integrated DNA Technologies or Invitrogen. 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 (Ct). The significance of differences between the group means was determined using the Mann-Whitney U test (n = 16). Student’s t test (n > 16), or χ² test (binomial data); p < 0.05 was considered significant.
Results
Diestrus-level E2 does not alter EAE disease
The hypothesis that E2 controls vitamin D3-mediated EAE resistance through regulation of vitamin D3 metabolism and/or VDR expression in the CNS predicts that E2 repletion would restore vitamin D3-mediated EAE resistance in OVX females. Therefore, we first determined an E2 repletion level that would not inhibit EAE in OVX mice independently of supplementary vitamin D3. Implanting 0.36 mg of E2 timed-release pellets into OVX B10.PL females inhibited EAE independently of vitamin D3 (44), so we repeated this study with 0, 0.1, or 0.36 mg of E2 implants. Two weeks after OVX and pellet implantation, the mice were immunized with MBP and monitored daily for clinical EAE signs. Uterine tissue was collected and weighed at the end of the study as an indicator of total E2 exposure (Fig. 1A).

The SHAM control mice had uterine weights of 0.11 ± 0.05 mg. The OVX mice with placebo pellets had lower uterine weights than did SHAM controls (Fig. 1A), confirming E2 depletion. The OVX mice with 0.1 mg of E2 pellets had uterine weights equal to the SHAM controls, indicating normal E2 levels. Finally, the OVX mice with 0.36 mg of E2 pellets had higher uterine weights than did SHAM controls, indicating high-level E2 repletion (44). Because high-level E2 repletion decreased the EAE incidence, peak severity, and cumulative disability independently of supplementary vitamin D3 (Fig. 1, B and C), but low-level E2 repletion alone had no effect, we used low-level E2 repletion in all subsequent experiments.

Vitamin D3 supplementation increased serum E2 in OVX mice
The next experiments investigated possible interactions between vitamin D3 and low-level E2 repletion in OVX mice. Female B10.PL mice were fed diets with (1 g/day; +D) or without (0 g/day; −D) vitamin D3. This +D diet provided 3-fold the amount of vitamin D3 in standard laboratory mouse chow. After 1 wk of −D or +D diet feeding, OVX or SHAM surgery was performed and 0 or 0.1 mg of E2 pellets was implanted. MBP immunizations were performed 2 wk after surgery. The serum E2 levels were quantified 7, 24, and 48 days after surgery, and uterine weights were measured 48 days after surgery (Fig. 2A). In the OVX/placebo mice, the E2 level declined significantly relative to the SHAM controls 24 days after surgery (p < 0.005), but rose by 48 days after surgery due to E2 synthesis by the adrenal glands (45). In the OVX/E2 mice, the E2 level was elevated 7 days after surgery (p < 0.005), but matched the SHAM controls 24 and 48 days after surgery. Unexpectedly, when the E2 data were analyzed by dietary vitamin D3 group, there was significantly more E2 in the +D OVX/placebo serum samples than in the −D OVX/placebo samples (p < 0.03) (Fig. 2B). This trend was also evident in the SHAM and OVX/E2 mice, but it did not reach significance due to the variability in serum E2 measurements. The uterine weights confirmed that the OVX mice had lower total E2 exposure than did the SHAM controls, whereas the 0.1 mg of pellet provided a total E2 exposure equal to the SHAM controls (Fig. 2C).

The serum 25-(OH)D3 levels were quantified at the end of the study. The groups ingesting the −D diet had significantly less serum 25-(OH)D3 than did the groups ingesting the +D diet (p < 0.01; Table I). The groups did not differ significantly with respect to terminal weights or serum calcium levels. These data are consistent with our published data (37), confirming that this level of vitamin D3 supplementation does not cause hypercalcemia.

FIGURE 1. Comparison of estrus and diestrus E2 levels on EAE induction in OVX female mice. A, Uterine weight. Groups of female B10.PL mice were ovariectomized and implanted s.c. with a 60-day release pellet containing 0, 0.1, or 0.36 mg of E2. Two weeks after surgery, the mice were immunized with MBP and evaluated daily for clinical EAE signs. Uterine tissue was collected at the end of the 25-day study. The means ± SD for six mice per group from one of two experiments are shown. ***p < 0.001 (Mann-Whitney U test). B, Clinical EAE severity. The experiment was performed as in A. The 0.36 mg of E2 group had significantly less severe EAE than did the placebo group from day 14 onward. C, Cumulative EAE disease. The cumulative EAE disease score was calculated by summing each animal’s daily EAE severity scores. The means ± SD for six mice per group are shown. *, p < 0.05 (Mann-Whitney U test).

Diestrus-level E2 enabled vitamin D3 to inhibit EAE in OVX females
Having confirmed that the serum E2 and 25-(OH)D3 levels differed between the respective pellet and dietary groups as intended, we next analyzed the EAE data. Consistent with our published report, the vitamin D3 significantly reduced the incidence 28% (p < 0.05), delayed disease onset by 10 days (p < 0.05), decreased the peak severity ~50% (p < 0.05), and diminished the cumulative disease severity ~70% (p < 0.05) in the SHAM females, but provided no benefits whatsoever in the OVX/placebo mice (Fig. 3 and Table II) (37). Extending our published results, we found that diestrus level E2 repletion restored all of the protective effects of vitamin D3 with respect to EAE incidence, onset, peak severity,
and cumulative disease, although it had no independent effect on EAE disease.

In B10.PL mice immunized with MBP, EAE follows a relapsing-remitting disease course (46), whereas in B6 mice immunized with MOG35-55 peptide, EAE follows a chronic progressive disease course (47). To learn whether E2 and vitamin D3 would cooperate to inhibit chronic progressive EAE as they did to inhibit relapsing-remitting EAE, we repeated the OVX experiments in B6 mice. Female B6 mice were fed placebo or E2 pellets and fed 0 or 1 μg/day vitamin D3, and uterine weights were measured 4 wk after MBP immunization. The composite means ± SD (≤8 mice/group) for one representative experiment of four are shown. *, p < 0.01 and **, p < 0.005 for comparisons to the SHAM controls (Mann-Whitney test).

### Table 1. Serum calcium and (OH)2D3 in SHAM and OVX mice with placebo or E2 implants and fed 0 or 1 μg/day vitamin D3

<table>
<thead>
<tr>
<th>Surgerya</th>
<th>Vitamin D3 (μg/day)</th>
<th>E2 Implant (mg)</th>
<th>Terminal Weight (g)</th>
<th>Serum Ca (mg/dl)</th>
<th>Serum 25-(OH)D3 (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM 1</td>
<td>0 0</td>
<td>0</td>
<td>19.9 ± 2.1</td>
<td>9.4 ± 1.5</td>
<td>17 ± 10**</td>
</tr>
<tr>
<td>SHAM 2</td>
<td>0 0</td>
<td>0</td>
<td>20.9 ± 2.2</td>
<td>17.0 ± 2.7</td>
<td>61 ± 29</td>
</tr>
<tr>
<td>SHAM 3</td>
<td>0 0</td>
<td>0</td>
<td>19.9 ± 2.7</td>
<td>8.2 ± 1.7</td>
<td>10 ± 8*</td>
</tr>
<tr>
<td>OVX</td>
<td>1 0</td>
<td>0.1</td>
<td>20.7 ± 1.6</td>
<td>10.2 ± 1.8</td>
<td>46 ± 6</td>
</tr>
<tr>
<td>OVX</td>
<td>0 0.1</td>
<td>0.1</td>
<td>21.2 ± 1.8</td>
<td>9.8 ± 1.8</td>
<td>14 ± 8**</td>
</tr>
<tr>
<td>OVX</td>
<td>1 0.1</td>
<td>0.1</td>
<td>21.8 ± 2.9</td>
<td>9.7 ± 1.2</td>
<td>64 ± 20</td>
</tr>
</tbody>
</table>

a Female B10.PL mice were fed diets with 0 or 1 μg/day vitamin D3 continuously beginning 1 wk before surgery. OVX or SHAM surgery was performed and a placebo or E2 pellet (0.1 mg; 60-day release) was implanted s.c. in each OVX mouse. Two weeks after surgery the mice were immunized to induce EAE as described in Materials and Methods.

b Serum Ca and 25-(OH)D3 were quantified at the end of the study as described in Materials and Methods. The data shown are the means ± SD (n=8 mice/group) for one experiment of four. *, p < 0.01 and **, p < 0.005 for comparisons to the SHAM controls (Mann-Whitney test).

In our previous study, vitamin D3 failed to inhibit MBP-induced EAE in the intact and castrated male B10.PL mice, indicating that male sex hormones did not impede vitamin D3-mediated EAE resistance (37). Here we examined whether E2 implants would enable vitamin D3-mediated EAE resistance in intact male mice. Male B6 mice were fed diets with 0, 1, or 2 μg/day vitamin D3, immunized with MOG35-55 peptide to model the progressive form
of MS often seen in men, and evaluated daily for EAE signs. The vitamin D$_3$-fed groups were not significantly different from the 0 µg/day vitamin D$_3$ controls for any EAE parameter (Table IV), consistent with our published data for male B10.PL mice (37). Implanting E$_2$ pellets in the males significantly increased their serum E$_2$ levels and inhibited EAE induction, as others reported previously (48). However, E$_2$ supplementation did not enable vitamin D$_3$ to further inhibit any EAE parameter in the males.

**Diestrus-level E$_2$ restored VDR gene expression and vitamin D$_3$ protection in OVX mice**

It is not clear why diestrus-level E$_2$ repletion restored the protective effects of vitamin D$_3$ in OVX females immunized to induce EAE. Two possibilities are E$_2$-mediated enhancement of in situ 1,25-(OH)$_2$D$_3$ accumulation through control of the Cyp27b1 or Cyp24a1 genes encoding the synthetic and inactivating enzymes, respectively, or E$_2$-mediated enhancement of the VDR in the CNS. The 1,25-(OH)$_2$D$_3$ synthesis and inactivation rates are proportional to the Cyp27b1 and Cyp24a1 transcripts, respectively (49). Therefore, to evaluate the propensity for hormone accumulation, we analyzed the Cyp27b1 and Cyp24a1 transcripts. As before, female B10.PL mice were fed +D or −D diets, OVX or SHAM surgery and placebo or E$_2$ pellet implantation was performed, and the mice were immunized with MBP. Ten days postimmunization (disease onset), spinal cord RNA was isolated and reverse transcribed, and transcripts were quantified by quantitative real-time PCR. Optimal PCR primers and reaction conditions were selected to amplify the transcripts with >90% efficiency (50). We recorded the threshold PCR cycles (Ct) at which the Cyp27b1 and Cyp24a1 amplicons were detected. The Ct is inversely proportional to transcript abundance, so the relative abundance of the two transcripts was expressed as ΔCt (Ct for Cyp24a1 minus the Ct for Cyp27b1) (Fig. 4A).

In nonimmunized mice, the Ct for Cyp24a1 was ~30 and the Ct for Cyp27b1 was ~28–29, so ΔCt was ~1–2, regardless of dietary vitamin D$_3$. Assuming equal amplification efficiencies and fluorescence intensities, this ΔCt indicates that Cyp27b1 transcripts were slightly more abundant than Cyp24a1 transcripts. From standard curves we estimated that the Cyp27b1-to-Cyp24a1 transcript ratio was ~4:1 in naive mice.

In MBP-immunized SHAM and OVX mice, the C$_t$ for Cyp24a1 shifted to ~32–33, while the C$_t$ for Cyp27b1 remained ~28–29, so ΔCt was ~3–4, regardless of dietary vitamin D$_3$ (Fig. 4A). This increase in the C$_t$ for Cyp24a1 from ~30 in naive mice to ~32–33 in MBP-immunized mice indicates that Cyp24a1 gene expression decreased with MBP immunization, increasing the Cyp27b1-to-Cyp24a1 transcript ratio to ~8:1. These data are consistent with our previous report showing decreased Cyp24a1 gene expression during CNS inflammation in female mice (37). There was a trend toward further reduction in Cyp24a1 gene expression in OVX mice with E$_2$ implants that did not reach significance. Thus, the Cyp27b1-to-Cyp24a1 transcript ratio might be >8:1 in these mice due to E$_2$-mediated Cyp24a1 suppression. Decreased Cyp24a1 gene expression during CNS inflammation would favor in situ 1,25-(OH)$_2$D$_3$ accumulation, consistent with the results presented in Table III.

We next investigated possible E$_2$-mediated enhancement of VDR gene expression in the CNS. Spinal cord samples collected 10 days postimmunization (disease onset) were analyzed for VDR and GAPDH transcripts. In samples from unimmunized controls, the C$_t$ for GAPDH and VDR were ~20 and ~29, respectively, regardless of dietary vitamin D$_3$. So, ΔC$_t$ (C$_t$ for VDR minus the C$_t$ for GAPDH) was ~9, indicating very low VDR gene expression (10–15 VDR copies/1000 GAPDH copies) (Fig. 4, B and C). In samples from the MBP-immunized SHAM mice ingesting the +D diet, the VDR C$_t$ decreased to ~24, indicating a ~32-fold increase in transcript abundance. This increase in VDR transcript abundance was also present in the MBP-immunized OVX mice with E$_2$ implants ingesting the +D diet, but it was absent in the −D SHAM group, the −D and +D OVX groups, and in the −D OVX group with E$_2$ implants. Thus, high-level VDR transcription in the spinal cord required an inflammatory stimulus, dietary vitamin D$_3$, and a source of E$_2$. Graphing the spinal cord VDR ΔC$_t$ at the time of EAE disease onset vs the cumulative EAE disease at the end of the 28-day study revealed a linear correlation between these parameters with an R$^2$ equal to 0.78 (Fig. 4D). This correlation was observed in two independent studies performed one year apart. We conclude that diestrus-level E$_2$ repletion restored the protective effects of vitamin D$_3$ in OVX females immunized to induce EAE, through decreased Cyp24a1 gene expression and increased VDR gene expression. These two actions enhanced in situ 1,25-(OH)$_2$D$_3$ accumulation and VDR function in the CNS, contributing to the resolution of inflammation and reduction of EAE disease.

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**FIGURE 3.** Vitamin D$_3$ inhibited EAE only in the intact females and the E$_2$-supplemented OVX females. A, EAE disease in SHAM, OVX, and OVX+/+E$_2$, females fed a diet with ( ●) or without (○) vitamin D$_3$. The experiment was performed as described in the Fig. 2 legend. Shown are the composite means ± SD for three independent experiments (10–23 mice/group). The SHAM−D and SHAM+/+D groups were significantly different from day 11 onward, and the OVX+/+E$_2$−D and OVX+/+E$_2$+/+D groups were significantly different from day 14 onward (p < 0.02; Mann-Whitney U test). B, Cumulative EAE disease in SHAM, OVX, and OVX+/+E$_2$, females fed a diet with (filled bars) or without (open bars) 1 µg/day vitamin D$_3$. The cumulative disease was calculated by summing each animal’s daily EAE disability scores for 28 days after MBP immunization. ∗, p < 0.05 (Mann-Whitney U test).
**Table II.** E2 repletion restored vitamin D3-mediated inhibition of EAE in OVX female mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Surgery</th>
<th>Dietary Vitamin D3 (μg/day)</th>
<th>E2 Implant (mg)</th>
<th>Incidence (%)</th>
<th>Onset (day)</th>
<th>Peak Severity</th>
<th>Cumulative Disease Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>B10.PL</td>
<td>SHAM</td>
<td>0</td>
<td>0</td>
<td>100 (25/25)</td>
<td>10 ± 3</td>
<td>2.3 ± 0.6</td>
<td>25.6 ± 9.2</td>
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<td>OVX</td>
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<td>0.1</td>
<td>100 (25/25)</td>
<td>13 ± 9</td>
<td>2.1 ± 0.4</td>
<td>20.9 ± 7.0</td>
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<tr>
<td></td>
<td>C57BL/6</td>
<td>SHAM</td>
<td>0</td>
<td>0</td>
<td>100 (13/13)</td>
<td>15 ± 2</td>
<td>1.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0</td>
<td>0.1</td>
<td>100 (13/13)</td>
<td>14 ± 1</td>
<td>1.7 ± 0.8</td>
<td>21.6 ± 8.1</td>
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<tr>
<td></td>
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<td>0.1</td>
<td>1</td>
<td>100 (88/88)</td>
<td>18 ± 4</td>
<td>1.4 ± 0.8</td>
<td>13.8 ± 9.0</td>
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</tbody>
</table>

* The experiment was performed as described in the Table I footnotes. The data are the composite means ± SD for two (C57BL/6) or four (B10.PL) independent experiments. The *p < 0.05 for comparisons between −D and +D mice within the same surgical and implant groups (Student’s t test).

**Discussion**

We have presented evidence that female mice with disrupted ovarian hormone production lost vitamin D3-mediated resistance to EAE and, conversely, that low-level E2 repletion restored vitamin D3-mediated resistance to EAE in OVX female mice. The low-level E2 repletion did not inhibit EAE disease independently of dietary vitamin D3. The vitamin D3 in the +D diet used here was 3-fold higher than laboratory chow, and it yielded serum 25-(OH)D3 levels that were ~1.6-fold higher than those in chow-fed mice. The vitamin D3-mediated EAE resistance was manifested as a lower incidence, later onset, decreased peak severity, and diminished cumulative disease severity in the MBP/B10.PL model, and as a diminished cumulative disease severity in the MOG/B6 model. None of these vitamin D3-mediated benefits were evident in male mice, with or without E2 supplementation. Our data showed that high-level VDR gene expression and function in the spinal cord required an inflammatory stimulus, E2, and sufficient dietary vitamin D3 to support in situ 1,25-(OH)2D3 synthesis. To our knowledge, this is the first evidence of synergy between a sex hormone and vitamin D3 in the control of an autoimmune disease, and the first evidence that E2 is essential for VDR gene expression and function in the inflamed CNS.

One mechanism of synergy between vitamin D3 and E2 appears to be vitamin D3 enhancement of E2 biosynthesis. We found that vitamin D3-supplemented OVX mice had 2-fold more serum E2 than did unsupplemented mice. Our data are consistent with published data showing that VDR-targeted female mice had uterine hypoplasia and impaired folliculogenesis, because lack of estrogen synthesis in the ovary decreased E2 biosynthesis (51). E2 supplementation reversed these defects. Moreover, 1,25-(OH)2D3 enhanced the transcription of the Cyp19 gene encoding estrogen synthase in glial cells (52) and placental trophoblasts (53). Estrogen synthesis (also termed aromatase), the rate-limiting enzyme in the formation of estrone and estradiol from the C19 androgens androstenedione and testosterone, is expressed in the gonads, adrenals, brain, and adipose tissue (45). Thus, 1,25-(OH)2D3 and VDR-dependent enhancement of E2 biosynthesis could be one mechanism allowing the estrogen and vitamin D3 endocrine systems to function synergistically in women.

A second mechanism of synergy between the estrogen and vitamin D3 endocrine systems appears to be E2 suppression of Cyp24a1 gene expression, leading to 1,25-(OH)2D3 accumulation, and enhancement of VDR gene expression in females. Our new data show E2-dependent transcriptional activation and function of the VDR gene in the spinal cord during an inflammation. This is true in other tissues as well. In osteoblasts, VDR gene expression decreased with E2 deprivation and increased with E2 supplementation (54, 55). Moreover, E2-mediated up-regulation of the VDR gene was also reported in the duodenal mucosa (56), where reduced VDR gene methylation correlated with transcriptional activation, elevated VDR protein, increased responsiveness to endogenous 1,25-(OH)2D3, and greater resistance to colonic carcinogenesis (57, 58). Liver cells (59) and breast cells (60, 61) also showed E2-mediated transcriptional activation of the VDR gene. In breast cells, an estrogen receptor (ER)-mediated mechanism controlled the VDR gene expression (60). Estrogen-responsive promoter elements were identified immediately upstream of exon 1c in the human VDR gene (61). Collectively, these data suggest that a second general mechanism of synergy between the estrogen and vitamin D3 endocrine systems is E2-mediated transcriptional activation of the VDR gene. Linking the first and second mechanisms together yields an amplification loop: 1,25-(OH)2D3

**Table III.** Spinal cord 1,25-(OH)2D3 in SHAM and OVX mice with placebo or E2 implants and fed 0 or 1 μg/day vitamin D3

<table>
<thead>
<tr>
<th>Surgery</th>
<th>Vitamin D3 (μg/day)</th>
<th>E2 Implant (mg)</th>
<th>Spinal Cord 1,25-(OH)2D3 (fmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM</td>
<td>0</td>
<td>0</td>
<td>44 ± 24</td>
</tr>
<tr>
<td>SHAM</td>
<td>1</td>
<td>0</td>
<td>226 ± 293</td>
</tr>
<tr>
<td>OVX</td>
<td>0</td>
<td>0</td>
<td>59 ± 22</td>
</tr>
<tr>
<td>OVX</td>
<td>1</td>
<td>0</td>
<td>189 ± 64**</td>
</tr>
<tr>
<td>OVX</td>
<td>0</td>
<td>0.1</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>OVX</td>
<td>1</td>
<td>0.1</td>
<td>178 ± 92**</td>
</tr>
</tbody>
</table>

* Female B6 mice were fed diets with 0 or 1 μg/day vitamin D3 continuously beginning 1 wk before surgery. OVX or SHAM surgery was performed and a placebo or E2 pellet (0.1 mg; 60-day release) was implanted s.c. in each OVX mouse. Two weeks after surgery the mice were immunized with MOG35-55 peptide.

**Spinal cord 1,25-(OH)2D3 were quantified at the end of the study. The data shown are the means ± SD (n = 6–7 mice/group) from one experiment of three. #, p < 0.005 for comparisons between −D and +D mice within the same surgical and implant groups (Mann-Whitney test).
E2-enhanced EAE resistance observed in SHAM females and E2-supplemented OVX females correlated with high levels of 1,25-(OH)2D3. This was necessary for E2 to inhibit EAE (73, 74). ER-β inhibition of EAE in mice with a targeted disruption of the VDR gene (72), whereas 1,25-(OH)2D3 did not (69). ER-β-mediated inhibition of EAE, we suggest that E2 and ER-β may enhance VDR expression in CD4+ T cells through estrogen-responsive promoter elements in the VDR gene (61).

An important question remaining to be answered is whether 1,25-(OH)2D3 exerts direct, VDR-independent protective effects in EAE, or only indirect effects attributable to increased E2 synthesis. Similarly unanswered is the question whether E2 exerts direct, VDR-independent protective effects in EAE, or only indirect effects attributable to increased VDR synthesis. There are reported differences in the mechanisms of action of these two hormones that suggest independent action. For example, 1,25-(OH)2D3 reversed the signs of severe acute EAE (41), whereas E2 did not (48). Also, E2 inhibited EAE in mice with a targeted disruption of the IL-10 gene (72), whereas 1,25-(OH)2D3 did not (69). ER-α expression was necessary for E2 to inhibit EAE (73, 74). ER-α is expressed in

Table IV. E2-supplementation did not enable vitamin D3 to inhibit EAE in intact male mice

<table>
<thead>
<tr>
<th>Dietary Vitamin D3 (µg/day)</th>
<th>E2 Implant (mg)</th>
<th>Serum E2 (pg/ml)</th>
<th>Incidence</th>
<th>Onset (day)</th>
<th>Peak Severity</th>
<th>Cumulative Disease Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>47 ± 9</td>
<td>100 (18/18)</td>
<td>17 ± 4</td>
<td>2.3 ± 1.0</td>
<td>20.6 ± 9.9</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>41 ± 22</td>
<td>100 (11/11)</td>
<td>19 ± 3</td>
<td>2.6 ± 1.4</td>
<td>18.1 ± 8.6</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>ND</td>
<td>100 (5/5)</td>
<td>14 ± 1</td>
<td>2.2 ± 0.3</td>
<td>20.4 ± 7.1</td>
</tr>
<tr>
<td>0.18</td>
<td></td>
<td>480 ± 2.64*</td>
<td>87 (7/7)</td>
<td>23 ± 5*</td>
<td>1.2 ± 0.7</td>
<td>8.9 ± 5.0***</td>
</tr>
<tr>
<td>1</td>
<td>0.18</td>
<td>371 ± 279*</td>
<td>100 (7/7)</td>
<td>21 ± 4</td>
<td>1.5 ± 0.6</td>
<td>13.6 ± 6.9</td>
</tr>
</tbody>
</table>

* Male B6 mice were fed a diet with 0, 1, or 2 µg/day vitamin D3 continuously beginning 1 wk before the placebo or E2 pellets were implanted. Two weeks postimplantation, the mice were immunized with MOG35-55 peptide. The data are the composite means ± SD for two independent experiments. #, p < 0.05 and **, p < 0.01 for comparisons within diet groups.

a Serum E2 was quantified at the end of the 28-day EAE study. Shown are the means ± SD for n = 5–8 mice/group.

b Incidence, day of onset, and cumulative disability were defined and evaluated as described in the Table 2 footnotes.

c Serum E2 was quantified at the end of the 28-day EAE study. Shown are the means ± SD for two independent experiments.

d Disease Score

FIGURE 4. The vitamin D3-mediated EAE resistance observed in SHAM females and E2-supplemented OVX females correlated with high level expression of VDR transcripts in the CNS. A. Relative Cyp24a1 and Cyp27b1 transcript abundance represented as ΔCt (Ct Cyp24a1 – Ct Cyp27b1). The experiment was performed as described in the Fig. 2 legend. Spinal cord samples were collected 10 days after MBP immunization. The RNA was isolated, reverse-transcribed, and real-time PCR was performed. The data are the means ± SD for one of two independent quantitative PCR analyses performed on samples from one of two independent experiments (n = 4–6 mice/group). For statistical analysis, the unprimed groups were compared with the SHAM MBP-primed groups. *, p < 0.05 (Mann-Whitney U test). B, VDR transcript abundance represented as ΔCt relative to GAPDH. C, VDR transcript abundance represented as copies per 1000 GAPDH copies. D, Correlation between cumulative EAE disease index and relative VDR transcript abundance represented as ΔCt relative to GAPDH.
neurons, glia, oligodendrocytes, lymphocytes, macrophages, and dendritic cells (34), but its expression in T lymphocytes was not necessary for E2-mediated inhibition of EAE (75). In contrast, our unpublished chimera data show that VDR expression is necessary in T lymphocytes for 1,25-(OH)2D3-mediated inhibition of EAE (C. G. Mayne, J. A. Spanier, and C. E. Hayes, unpublished data). These hormone receptor expression studies suggest that 1,25-(OH)2D3 may primarily target T lymphocytes, whereas E2 may primarily target non-T cells. Thus, although the two hormones E2 and 1,25-(OH)2D3 show many similar mechanisms of action suggesting synergy between them, they also show significant differences in mechanism suggesting each hormone can act independently. Additional experiments are underway to define the synergistic and independent functions of E2 and 1,25-(OH)2D3 more precisely.

The functional synergy between the vitamin D3 and E2 endocrine systems may be a driver for the relapsing-remitting MS disease phenotype. Seasonal fluctuations in UV light exposure and thus circulating 25-(OH)D3 levels show the same periodicity as seasonal fluctuations in MS attacks (15–21). The fact that seasonal changes in UV light/vitamin D3 preceded changes in MS attack rates by ~3–4 mo suggests a causal link between UV light/vitamin D3 fluctuations and changes in the MS attack rates. Data showing beneficial effects of 25-(OH)D3 only in females is also consistent with this hypothesis (37, 38). Seasonal increases in UV light exposure and serum 25-(OH)D3 and spinal cord 1,25-(OH)2D3 levels could trigger the protective amplification loop described above. The increased E2 biosynthesis and E2-mediated enhancement of VDR expression and function would enable 1,25-(OH)2D3 and E2 to activate antiinflammatory mechanisms that drive MS disease into remission. Conversely, seasonal declines in UV light exposure and serum 25-(OH)D3 and spinal cord 1,25-(OH)2D3 levels could interfere with the amplification loop and allow inflammatory mechanisms to ignite and precipitate MS attacks.

The functional synergy between the vitamin D3 and E2 endocrine systems may also be a driver of the increasing female bias in MS. An equal number of women and men were afflicted with MS until about 1950, when prevalence surveys first revealed a 1.4:1 female bias (76). Recent estimates put the sex ratio between ~3:1 (28) and ~5:1 (30), depending on the decade of birth. An increase in the female bias of MS has been observed in the United States (26, 77), Canada (28, 30), Australia (78), the United Kingdom (29, 79), Norway (27, 80), and Sardinia (81), due to an increased incidence among women, rather than to a decreased incidence among men. One explanation for the female bias could be X-linked risk factors, but extensive X chromosome linkage studies have not revealed MS susceptibility loci (82), and the rapidity of the female-to-male sex ratio increase argues against a genetic origin (28). Another explanation for the female bias in MS invokes sex hormone-determined differences in immune responsiveness (25). This explanation is consistent with data showing that the female bias becomes apparent only after sexual maturity (31), but it is not consistent with data showing a rapid female-specific increase in MS risk (26–30, 77–81).

The proposed functional synergy between the vitamin D3 and E2 endocrine systems could explain the rapidly increasing female-to-male sex ratio in MS. The female bias in MS would become evident at puberty, because with increasing E2 biosynthesis would come increasing responsiveness to sunlight and vitamin D3 via the amplification loop, with E2 enhancing 1,25-(OH)2D3 synthesis and function by ER-mediated down-regulation of Cyp24a1 and up-regulation of VDR, and 1,25-(OH)2D3 enhancing E2 biosynthesis by VDR-mediated up-regulation of estrogen synthase. Because high UV exposure and vitamin D3 supplies apparently reduce the MS risk in women but not in men (37, 38), it follows that diminishing sunlight exposure and vitamin D3 supplies due to lifestyle changes could drive a disproportional increase in MS among women, establishing a female gender bias. Moreover, the lifestyle changes that have decreased overall UV light exposure and serum 25-(OH)D3 levels in women over the last half century (83) could be driving the rapidly increasing female gender bias. Examples of significant lifestyle changes are increased numbers of women in the workforce, decreased outdoor activity, increased sun avoidance, and use of sunscreens (which inhibit vitamin D3 biosynthesis). We suggest that the vitamin D3 and E2 functional synergy hypothesis, rather than the X-chromosome or sex hormone hypothesis, is most consistent with recent data on the rapid rise in MS among women.

We have proposed that there is functional synergy between the vitamin D3 and E2 endocrine systems, and that this synergy is causally related to the relapsing-remitting MS disease phenotype and the increasing female bias in MS prevalence. Our data and the mechanisms we have proposed to explain the data have very significant implications for MS. Specifically, inadequate sunlight exposure and low vitamin D3 supplies may undermine the beneficial effects of estrogens, to the extent that these activities depend on enhancement of VDR expression and function. Moreover, inadequate E2 biosynthesis due to ovarian failure or menopause may undermine the beneficial effects of vitamin D3, to the extent that these activities depend on enhancement of E2 synthesis. Combined vitamin D3 and E2 deprivation, such as one might expect for older female MS patients with limited mobility, could have a devastating synergistic effect, triggering the evolution of relapsing-remitting MS into a chronic-progressive disease course with rapid accumulation of disability.

MS is a devastating neurodegenerative disease that imposes heavy burdens on patients, on families, and on health care systems throughout the world. At an estimated lifetime cost in excess of $2.2 million per MS case, the implications of the sustained increases in female cases to the world’s strained health care systems are staggering (28). In this context, it is encouraging that modifiable environmental factors appear to set the disease threshold and may hold the key to preventing the vast majority of MS cases (6). Sunlight exposure and vitamin D3 supplies appear to be those modifiable environmental risk factors (9, 13). If health care providers were to monitor serum 25-(OH)D3 levels, especially in girls and women who are genetically related to an individual with MS, and prescribe enough sunlight exposure and/or vitamin D3 supplementation to maintain >100 nmol/L of serum 25-(OH)D3 throughout the year, an estimated 90% of MS cases might be prevented (15). For men and women already afflicted with MS, intermittent 1,25-(OH)2D3 pulse dose therapy (F. E. Nashold, R. A. Derks, and C. E. Hayes, manuscript in preparation) in the context of sufficient natural E2 in young women or E2 replacement therapy in postmenopausal women might activate antiinflammatory mechanisms that drive MS disease into remission and significantly decrease the cumulative disability. The overwhelming body of evidence suggests that these intervention strategies could dramatically reduce the impact of MS on patients, on families, and on our health care systems.

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Disclosures
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References