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

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

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-estradiol (E₂) is essential for vitamin D₃-mediated protection, ovariectomized female mice were given E₂ or placebo and evaluated for vitamin D₃-mediated EAE resistance. Diestrus-level E₂ implants alone provided no benefit, but they restored vitamin D₃-mediated EAE 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 D₃ 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 Journal of Immunology, 2009, 183: 3672–3681.

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 D 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 $\sim 3.4 \times 10^{-9}$, arguing strongly for a cause-effect relationship between Cyp27b1 mutation, reduced 1,25-(OH)₂D₃, and MS.
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)\(_2\)D\(_3\) 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)\(_2\)D\(_3\) and 1,25-(OH)\(_2\)D\(_3\) 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 D\(_3\). High-level vitamin D\(_3\) supplementation inhibited EAE in intact adult female mice, but not in ovariectomized (OVX) females or males (37). Thus, vitamin D\(_3\) provided a female-specific and sex hormone-dependent protective effect in EAE. Interestingly, high serum 25-(OH)\(_2\)D\(_3\) levels correlated with a reduced MS risk in women but not in men (38). If vitamin D\(_3\) 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 D\(_3\) insufficiency would increase MS risk in a female-specific manner.

Here, we investigated the female-specific and sex hormone-dependent protective effect of vitamin D\(_3\) in EAE in more detail. To test the hypothesis that 17β-estradiol (E\(_2\)) might regulate vitamin D\(_3\) metabolism and/or VDR expression in the CNS, OVX female mice were implanted with E\(_2\) or placebo pellets and evaluated for vitamin D\(_3\)-mediated EAE resistance, vitamin D\(_3\) metabolism, and VDR expression in the CNS. Our data show for the first time that E\(_2\) enables vitamin D\(_3\) 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 D\(_3\)-based strategies to decrease MS risk and disease severity.

**Materials and Methods**

**Mice**

The B10.PL-H\(^2\)H\(^2\)-T18a/(73NS)/SnJ (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 mg/day of vitamin D\(_3\) 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 D\(_3\) exactly as we described (37). The vitamin D\(_3\) (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 \(0 \sim D\) diet or 1 \(\mu\)g/day \(\sim D\) diet of vitamin D\(_3\) 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 \(\sim D\) 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.
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 µg/day; +D) or without (0 µg/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](http://www.jimmunol.org/DownloadedFrom/3674ESTROGEN_CONTROLS_VITAMIN_D-MEDIATED_EAE_RESISTANCE)

**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 ovariec‐tomized 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 < 0.001** (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,
FIGURE 2. Implantation of diestrus level E2 pellets restored serum E2 and uterine weights to normal levels in OVX mice. A. Serum E2 concentration 7, 24, and 48 days after pellet implantation. Female B10.PL mice were fed diets with 0 or 1 μg/day vitamin D3. One week later, 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 MBP. Serum E2 levels were quantified by ELISA. The composite means ± SD were calculated for at least 11 mice per group from four independent experiments. ***, p < 0.005 for comparisons to the SHAM controls (Mann-Whitney U test). B. Relationship between dietary vitamin D3 and serum E2 in MBP-immunized mice. The experiment was performed as in A. The serum E2 levels were quantified 24 days postsurgery (10 days postimmunization). The means ± SD (n = 6–8 mice/group) for one representative experiment of four are shown. ***, p < 0.005 and *, p < 0.03 for the indicated comparisons (Mann-Whitney U test). C. Uterine weight. The experiment was performed as in A. The uterine weights were measured 4 wk after MBP immunization. The composite means ± SD for four independent experiments are shown (SHAM/−D/D−E2, n = 26; SHAM/−D/+E2, n = 35; OVX/−D−E2, n = 29; OVX/+D−E2, n = 27; OVX/−D+/E2, n = 12; OVX/+D+/E2, n = 13). For statistical analysis, each OVX group was compared with the SHAM control group ingesting the same diet. ***, p < 0.01 and ***, p < 0.001 (Mann-Whitney U test).

Table 1. Serum calcium and 25-(OH)D3 in SHAM and OVX mice with placebo or E2 implants and fed 0 or 1 μg/day vitamin D3

<table>
<thead>
<tr>
<th>Vitamin D3 (μg/day)</th>
<th>E2 Implant (mg)</th>
<th>Terminal Weight (g)</th>
<th>Serum Caa (mg/dl)</th>
<th>Serum 25-(OH)D3b (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM/0 0</td>
<td>0.0 ± 1.1</td>
<td>4.5 ± 0.5</td>
<td>2.9 ± 0.2</td>
<td>24.6 ± 1.2</td>
</tr>
<tr>
<td>SHAM/1 0</td>
<td>0.0 ± 1.1</td>
<td>4.5 ± 0.5</td>
<td>2.9 ± 0.2</td>
<td>24.6 ± 1.2</td>
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<td>OVX/0 0</td>
<td>0.0 ± 1.1</td>
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<td>OVX/1 0</td>
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<td>4.5 ± 0.5</td>
<td>2.9 ± 0.2</td>
<td>24.6 ± 1.2</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 = 6–7 mice/group) from one experiment of four. *, p < 0.01 and **, p < 0.005 for comparisons to the +D SHAM controls (Mann-Whitney test).

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 −D or −D diets, OVX, or SHAM surgery was performed, and 0 or 0.1 mg of E2 pellets was implanted in the OVX mice. The MOG35–55 immunizations were performed 2 wk after surgery. In the SHAM B6 mice, the vitamin D3 reduced the cumulative disease severity by 43% (p < 0.05) compared with the −D SHAM group, and there were trends toward lower incidence and decreased peak severity that did not reach significance (Table II). However, the vitamin D3 provided no benefits whatsoever in the OVX/placebo B6 mice. Comparing the OVX B6 mice with 0.1 mg of E2 implants to the OVX B6 mice with placebo implants, we saw a delay in disease onset and a 36% reduction in the cumulative disease (p < 0.02) that were attributable to the E2 implants independently of dietary vitamin D3. However, comparing the +D OVX/E2 mice to the −D OVX/E2 mice, we found that the +D diet further reduced the cumulative disease by 37% (p < 0.001), and there was a trend toward lower peak severity. The animals ingesting the +D diet had significantly higher spinal cord 1,25-(OH)2D3 levels than did the animals ingesting the −D diet (p < 0.005; Table III), although the serum 1,25-(OH)2D3 levels were not different between the groups (data not shown). These data confirm our report that 1,25-(OH)2D3 synthesis occurred in the inflamed spinal cord, independently of 1,25-(OH)2D3 synthesis in the kidney (37). In summary, E2 repletion provided a benefit that was independent of dietary vitamin D3 in B6 mice, and additionally, E2 repletion restored the ability of dietary vitamin D3 to inhibit EAE disease.

E2 supplementation did not enable vitamin D3 to inhibit EAE in intact male mice

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 Cyp27bl and 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 Cyp27bl and Cyp24a1 transcripts, respectively (49). Therefore, to evaluate the propensity for hormone accumulation, we analyzed the Cyp27bl 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 Cyp27bl 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 Cyp27bl) (Fig. 4A). In nonimmunized mice, the Ct for Cyp24a1 was ~30 and the Ct for Cyp27bl 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 Cyp27bl transcripts were slightly more abundant than Cyp24a1 transcripts. From standard curves we estimated that the Cyp27bl-to-Cyp24a1 transcript ratio was ~4:1 in naive mice.

In MBP-immunized SHAM and OVX mice, the Ct for Cyp24a1 shifted to ~32–33, while the Ct for Cyp27bl remained ~28–29, so ΔCt was ~3–4, regardless of dietary vitamin D$_3$ (Fig. 4A). This increase in the Ct 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 Cyp27bl-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 Cyp27bl-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 Ct for GAPDH and VDR were ~20 and ~29, respectively, regardless of dietary vitamin D$_3$. So, ΔCt (Ct for VDR minus the Ct 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 Ct 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 ΔCt 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.
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 a lack of estrogen synthase 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 synthase (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 Cyp2a1 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 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 a (%)</th>
<th>Onset c (day)</th>
<th>Peak Severity</th>
<th>Cumulative Disease Score d</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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<tr>
<td></td>
<td>OVX</td>
<td>0</td>
<td>0</td>
<td>100 (30/30)</td>
<td>11 ± 4</td>
<td>2.0 ± 0.5</td>
<td>22.1 ± 11.5</td>
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<tr>
<td></td>
<td>OVX</td>
<td>0</td>
<td>0</td>
<td>100 (39/39)</td>
<td>11 ± 4</td>
<td>2.4 ± 1.0</td>
<td>20.9 ± 7.1</td>
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<tr>
<td></td>
<td>OVX</td>
<td>0.1</td>
<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>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1</td>
<td>0.1</td>
<td>72 (18/25)</td>
<td>26 ± 10*</td>
<td>0.9 ± 0.2*</td>
<td>12.4 ± 4.9*</td>
</tr>
<tr>
<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>
<td>20.9 ± 9.6</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>1</td>
<td>85 (11/13)</td>
<td>16 ± 3</td>
<td>1.3 ± 0.8</td>
<td>12.0 ± 9.6*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0</td>
<td>100 (13/13)</td>
<td>14 ± 1</td>
<td>1.7 ± 0.8</td>
<td>21.6 ± 8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1</td>
<td>100 (13/13)</td>
<td>14 ± 1</td>
<td>2.4 ± 0.7</td>
<td>29.6 ± 8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>0.1</td>
<td>100 (88/88)</td>
<td>18 ± 4</td>
<td>1.4 ± 0.8</td>
<td>13.8 ± 9.0*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OVX</td>
<td>1</td>
<td>86 (67/67)</td>
<td>19 ± 3</td>
<td>1.0 ± 0.6</td>
<td>8.7 ± 5.7</td>
<td></td>
</tr>
</tbody>
</table>

a 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).

b Mice with a cumulative EAE severity score ≥1 were considered to have EAE. Numbers of mice are given parenthetically. *p < 0.05 (χ² test).

c The day of onset was recorded as the day a mouse first had a cumulative EAE score ≥1.

d Each animal’s daily EAE disability scores were summed for 28 days postimmunization. #p < 0.02 for the comparison between the E₂-repleted and placebo-repleted groups ingesting a diet without vitamin D₃. **p < 0.001 for the comparison between the E₂-repleted and placebo-repleted groups ingesting a diet with vitamin D₃.

### 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 b (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>0.1</td>
<td>0</td>
<td>29 ± 5</td>
</tr>
<tr>
<td>OVX</td>
<td>1</td>
<td>0.1</td>
<td>178 ± 92**</td>
</tr>
</tbody>
</table>

a 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.

b Spinal cord 1,25-(OH)₂D₃ were quantified at the end of the study. The data shown are the means ± SD (n=7–8 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).
enhances E2 biosynthesis by VDR-mediated up-regulation of estrogen synthase, and E2 enhances 1,25-(OH)2D3 synthesis and function by ER-mediated down-regulation of Cyp24a1 and up-regulation of VDR. These two mechanisms and possibly others allow the vitamin D and estrogen endocrine systems to function synergistically in women. Large increases in maternal serum estradiol and 1,25-(OH)2D3 levels occur during pregnancy due to placental production of these hormones (62). Thus, the synergy between the vitamin D and E2 endocrine systems may have evolved to support successful reproduction through maternal immune tolerance for fetal Ags.

The lack of interaction between E2 and vitamin D3 in males was puzzling, since males express ER, and E2 is an effective regulator of EAE in males (63). We suspect that there may be differential regulation of the Cyp24a1 gene between males and females. We previously reported that males expressed higher levels of Cyp24a1 mRNA in the CNS than do females, and they did not show complete repression of Cyp24a1 gene expression during inflammation, as did females (37). This resulted in a failure to accumulate 1,25-(OH)2D3, which could undermine benefits derived from E2-mediated enhancement of VDR expression.

The synergy between 1,25-(OH)2D3 and E2 as inhibitors of EAE was necessary for E2 to inhibit EAE (73, 74). ER-mediated inhibition of EAE, or only indirect effects attributable to increased E2 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...
neurons, glia, oligendrocytes, 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 supports 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. An equal number of women and men were afflicted with MS, and the female/male sex ratio increase argues against a genetic origin (28). Another explanation for the female bias in MS invokes sex hormone differences 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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is associated with decreased CpG island methylation and increased mRNA and protein expression of the colonic vitamin D receptor. Oncol. Res. 11: 255–264.


