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Research Article

Neuroprotection in Cerebral Cortex Induced by the Pregnancy Hormone Estriol

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ABSTRACT

In multiple sclerosis (MS), demyelination occurs in the cerebral cortex, and cerebral cortex atrophy correlates with clinical disabilities. Treatments are needed in MS to induce remyelination. Pregnancy is protective in MS. Estriol is made by the fetoplacental unit, and maternal serum estriol levels temporally align with fetal myelination. Here, we determined the effect of estriol treatment on the cerebral cortex in the preclinical model of MS, experimental autoimmune encephalomyelitis (EAE). Estriol treatment initiated after disease onset decreased cerebral cortex atrophy. Neuropathology of the cerebral cortex showed increased cholesterol synthesis proteins in oligodendrocytes, more newly formed remyelinating oligodendrocytes, and increased myelin in estriol-treated EAE mice. Estriol treatment also decreased the loss of cortical layer V pyramidal neurons and their apical dendrites and preserved synapses. Together, estriol treatment after EAE onset reduced atrophy and was neuroprotective in the cerebral cortex.

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Introduction

Gray matter (GM) atrophy is a strong indicator of disability progression in multiple sclerosis (MS), an autoimmune-mediated neurodegenerative disease of the central nervous system. It is well established that GM atrophy in MS is closely tied to clinical disability.[1,2] In fact, GM atrophy is better at predicting motor disability than either white matter atrophy or white matter lesion load.[2,3] Cortical GM atrophy, in particular, has been associated with both motor[4-6] and cognitive disability.[6-8] Cortical GM atrophy occurs early in MS and may become more rapid in progressive MS.[4,8] However, little is known about the mechanisms underlying cortical GM atrophy. Since white matter inflammation does not fully predict cortical atrophy, this suggests an at least partially independent neurodegenerative process occurring within cortical GM.[9] Cortical lesions are characterized by cortical demyelination and may contribute to cortical GM atrophy,[10] although it was also reported that cortical thinning can occur in areas beyond cortical lesions.[11]

Currently available disease-modifying treatments have robust immunomodulatory effects on the peripheral immune system and

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reduce relapses. However, a major unmet need is to develop treatments to induce remyelination and neuroprotection within the central nervous system.

Estriol, a hormone produced by the fetoplacental unit during pregnancy, is a promising neuroprotective treatment for MS. Pregnancy is protective in MS. Decreased relapses occur in the third trimester when maternal serum estriol levels are at their highest,[12] and multiparity is associated with decreased disability.[13-15] Pregnancy is also when myelination begins, and synaptic plasticity occurs in the fetal central nervous system. In phase 2 clinical trials, estriol treatment in MS showed anti-inflammatory effects with reduced gadolinium-enhancing lesions, immunomodulation in the peripheral immune system,[16,17] and reduced relapses.[18-21] Direct neuroprotective effects were also suggested, since estriol-treated subjects had less cortical GM atrophy, particularly in those with no enhancing lesions. In addition, reduced cerebral cortex atrophy correlated with improvement in cognitive testing performance.[18-20,22] Determining the mechanisms underlying the neuroprotective effects of estriol treatment in the cerebral cortex is challenging in humans but can be addressed in MS animal models.

Chronic experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice is a valuable tool for investigating neurodegenerative mechanisms underlying GM pathology in MS. GM atrophy occurs in this EAE model in conjunction with demyelination, axonal damage, microglial activation, synaptic loss, and neuronal loss[23-25] as reviewed.[26] Estriol treatment in EAE induces a beneficial immunomodulatory effect in the peripheral immune system, reduces spinal cord pathology, and ameliorates walking disability.[27,28] Furthermore, estriol has been shown to preserve hippocampal CA1 volume loss and prevent decreases in synaptic number and transmission in the hippocampus of EAE mice.[29] Here, we determined whether therapeutic estriol treatment after disease onset can reduce cortical atrophy by magnetic resonance imaging (MRI) in EAE and reduce cortical pathology therein.

To that end, we combined the use of in vivo MRI, clear lipid-exchanged acrylamide-hybridized rigid imaging-compatible tissue-hydrogel (CLARITY), and immunohistochemistry to determine the effect of estriol treatment on neurodegenerative mechanisms within the cerebral cortex during EAE in C57BL/6 mice as well as in informative mouse lines, namely Thy1-YFP$^+$ mice that express a yellow fluorescent protein (YFP) in cortical layer V pyramidal neurons, their projections and apical dendrites,[30] and Cspg4-CreERT2/Mapt-mGFP mice that express green fluorescent protein (GFP) in newly formed remyelinating oligodendrocytes.[31]

Materials and Methods

Animals

All mice (8-16 weeks old) used in this study were from the C57BL/6J background, originally ordered from Jackson Labs (Bar Harbor), and bred within our facilities. Twenty female Thy1-YFP$^+$ C57BL/6J mice were used in the therapeutic estriol treatment study. This experiment was validated with 2 independent cohorts of 21 and 15 mice, respectively. Twenty-four female wild-type C57BL/6J mice were used in an experiment to assess the effect of ERβ ligand on cortical pathology. This experiment was validated with an independent cohort of 19 mice. Nineteen female Cspg4-CreERT2/Mapt-mGFP mice were used to investigate cortical remyelination with ERβ ligand treatment in EAE. This experiment was validated with an independent cohort of 21 mice. Cspg4-CreERT2/Mapt-mGFP mice were obtained by crossing B6.Cg-Tg(Cspg4-cre/Esr1*)BAkik/J with B6;129P2-Mapt<tm2Arbr>/J mice.[31,32] The estriol pretreatment study used 28 female Thy1-YFP$^+$ C57BL/6J mice (Supplementary Fig. S1).

Experimental Autoimmune Encephalomyelitis Induction and Experimental Treatment

EAE was induced as described previously.[23] EAE was scored on a scale of 0 through 5 as described previously.[33]

In the therapeutic estriol treatment experiment, either a 90-day release pellet of estriol at a 5 mg dose or a placebo pellet (Innovative Research of America) was implanted after mice showed symptoms of EAE as described previously.[27] In the pretreatment experiment (Supplementary Fig. S1), either an estriol or placebo pellet was similarly implanted 1 week prior to EAE induction. This dose of estriol was previously shown to induce a serum level of estriol approximating late pregnancy in mice.[27]

For ERβ ligand treatment, diarylpropionitrile (Tocris) was administered as described previously.[32,33] For Cspg4-CreERT2/Mapt-mGFP transgenic mice, tamoxifen (Sigma-Aldrich) was administered subcutaneously 2 weeks prior to ERβ ligand treatment for 5 consecutive days, as described previously.[32,33]

Magnetic Resonance Imaging

All animals were scanned in vivo at the Ahmanson-Lovelace Brain Mapping Center at University of California, Los Angeles, on a 7T Bruker imaging spectrometer (Br2ker Instruments), as described previously.[23]

Atlas-Based Morphometry

A minimum deformation atlas was created using the magnetic resonance images from all subjects. The cerebral cortex was manually labeled on the minimum deformation atlas using BrainSuite 18a[34] (http://brainsuite.org) as described previously.[24] The labels were warped to the individual images and manually corrected by an investigator blind to the disease and treatment group (C.E.M.).

Clear Lipid-Exchanged Acrylamide-Hybridized Rigid Imaging-Compatible Tissue-Hydrogel

The brains and spinal cords from the pretreatment experiment and the spinal cords from the therapeutic treatment experiment were optically cleared using the CLARITY protocol modified for passive clearing, as described previously.[25]

Immunohistochemistry

Sagittal brain sections from a subset of animals were stained using immunohistochemistry, as described previously.[23] Tissues were stained for postsynaptic density protein 95 (PSD95; Millipore), myelin basic protein (MBP; Aves), green fluorescent protein (GFP; Abcam), glutathione S-transferase pi (GSTΠ; Enzo Life Sciences), major histocompatibility complex II (MHCII; BioLegend), ionized calcium-binding adapter molecule 1 (Iba1; Wako), anti-adenomatous polyposis coli clone (CC1; Calbiochem),

farnesyl diphosphate synthase (FDPS; Invitrogen), or hydroxymethylglutaryl-CoA synthase (HMGCS1; Invitrogen).

Microscopy

Laser scanning confocal microscopy for CLARITY analysis was performed at the California NanoSystems Institute Advanced Light Microscopy/Spectroscopy Shared Resource Facility at University of California, Los Angeles, as described previously.[25] Immunostained sections were imaged using an BX51 fluorescence microscope with a DP50 digital camera (Olympus).[24] ImageJ (https://fiji.sc) was used to analyze images. In all analyses, the investigator was blind to the treatment group and focused on the sensorimotor cortex (Fig. 2A).

Statistical Analyses

Atlas-based morphometry, CLARITY, and immunohistochemistry data were analyzed in R (https://www.r-project.org). Two-group comparisons were conducted using a Mann-Whitney *U* test (2-tailed). Regression analyses are reported as Pearson correlation coefficients. All reported *P* values are corrected for multiple comparisons by controlling for the false discovery rate[35] within each experiment. Confidence intervals of 95% were found by resampling (10,000 bootstraps).

Results

Estriol Treatment After Disease Onset Spares Cortical Atrophy in Experimental Autoimmune Encephalomyelitis

We used in vivo MRI to investigate whether cortical atrophy during chronic EAE could be affected by treatment with estriol at a dose that induces a pregnancy level in serum.[27] Estriol-treated EAE mice, placebo-treated EAE mice, and placebo-treated healthy control mice were imaged and sacrificed 45 days after disease induction. Estriol treatment initiated after EAE onset reduced clinical severity scores (Fig. 1). As a baseline, placebo-treated EAE mice compared to placebo-treated healthy controls ($P = .0047$) (Fig. 2A, B) demonstrated a 9.3% reduction in cerebral cortex volume, consistent with previous natural history studies in untreated EAE.[23,25] Importantly, estriol-treated EAE mice had significantly reduced cerebral cortex atrophy (higher cortical volumes) as compared to placebo-treated EAE mice ($P = .012$) (Fig. 2A, B).

Estriol Treatment Reverses Neurodegeneration in the Cerebral Cortex

Next, we investigated cellular mechanisms underlying the estriol-mediated protection from cortical atrophy. We quantified cortical layer V pyramidal neurons in the same mice and region used for in vivo MRI analysis (Fig. 2A). The mice used were Thy1-YFP^{+}, which expressed YFP in cortical layer V pyramidal neurons and projections.[30] As a baseline, placebo-treated EAE mice demonstrated a 16.1% decrease in cortical layer V neurons compared to healthy controls ($P = .0047$) (Fig. 2C, D). Interestingly, cortical layer V neurons were preserved in estriol-treated EAE mice compared to placebo-treated EAE mice ($P = .0047$) (Fig. 2C, D) and, indeed, were not significantly different than healthy controls. We next examined synaptic integrity in the cerebral cortex because synapse loss is known to occur in MS[36] and EAE.[29,37] Dendritic spine density on the apical dendrites of cortical layer V pyramidal neurons showed a reduction in placebo-treated EAE mice versus healthy controls ($P = .0080$) (Fig. 2E, F). In contrast, estriol-treated EAE mice demonstrated sparing of spine density compared to placebo-treated EAE mice ($P = .044$) (Fig. 2E, F) and were not

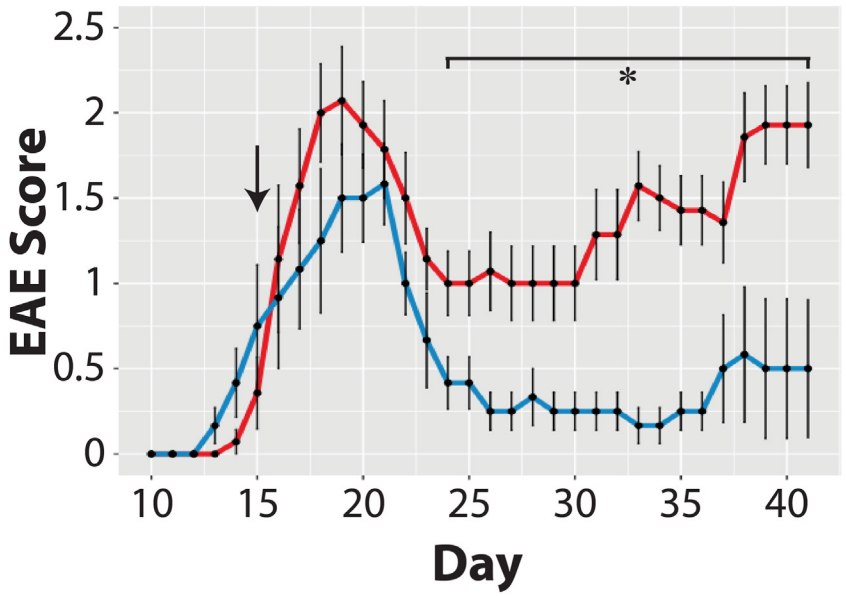

Figure 1.
Therapeutic estriol treatment reduces clinical scores. Estriol (blue; n = 7) or placebo pellets (red; n = 6) were implanted in each animal at first sign of disease. The average day of onset was 15 days after disease induction (arrow). Estriol-treated mice demonstrated significantly reduced EAE severity compared with placebo-treated mice 23 days after disease induction. The ameliorative effect continued for 45 days after disease induction when the animals were sacrificed. A one-way ANOVA indicated a significant effect of estriol treatment ($F[1,62] = 22.85$; $P = .000011$). The asterisk indicates a significant difference ($P < .05$, corrected for false discovery rate) in EAE scores between placebo-treated and estriol-treated EAE mice. EAE, experimental autoimmune encephalomyelitis.

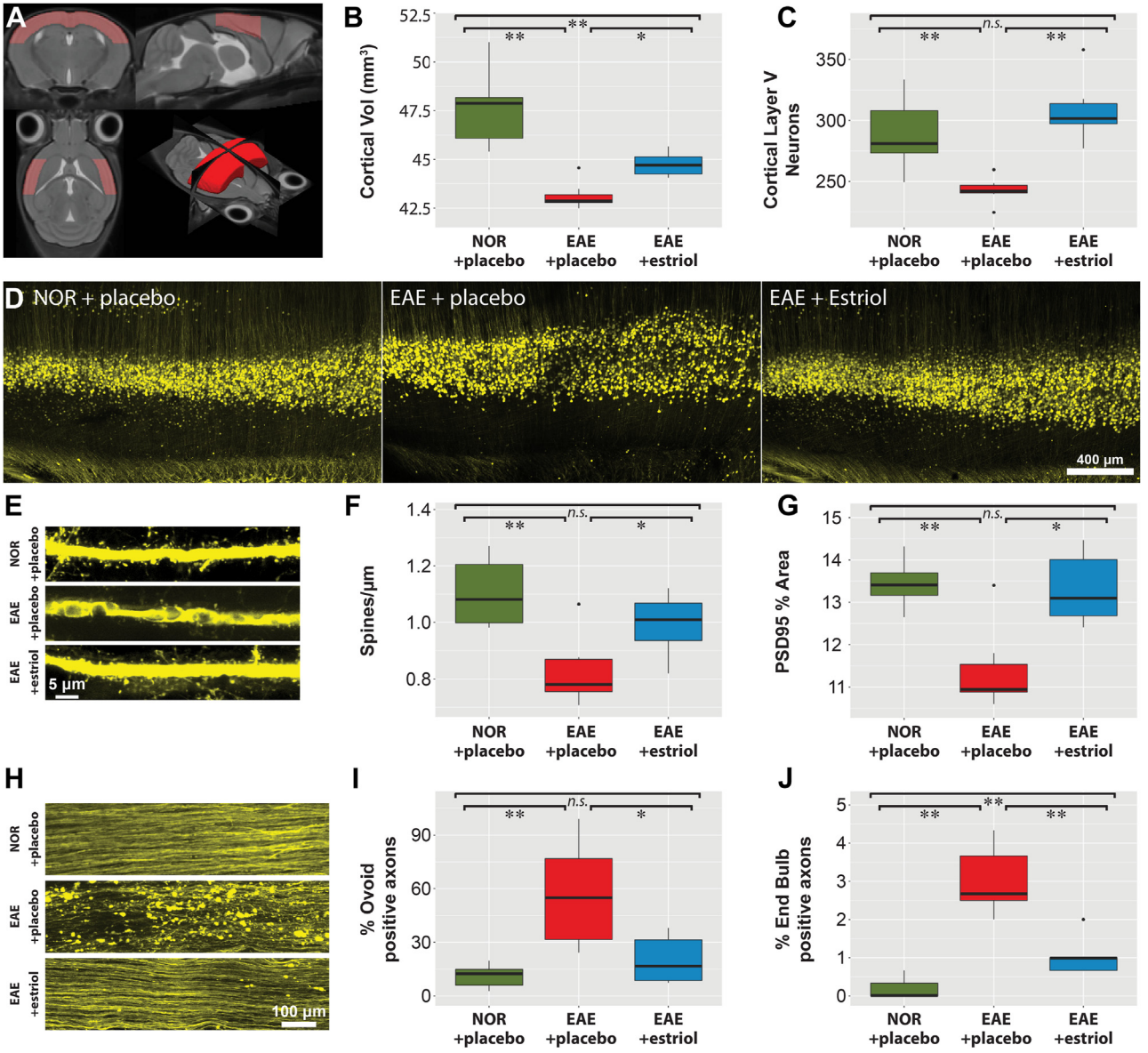

Figure 2.

Estriol treatment after disease onset reduces cortical gray matter atrophy and mitigates underlying neurodegeneration in the cerebral cortex during EAE. In vivo, MRI was collected at day 45 from Thy1-YFP$^+$ mice with EAE treated with placebo (EAE + placebo, red, n = 6), with EAE and treated with estriol (EAE + estriol, blue, n = 7) healthy controls treated with placebo (NOR + placebo, green, n = 7) for pathological analysis. (A) A minimum deformation atlas was constructed from all the images in the data set, and the cerebral cortex was delineated on the minimum deformation atlas and warped to the constituent images. (B) Significant atrophy was observed in placebo-treated EAE mice compared to placebo-treated healthy control mice. Estriol-treated EAE mice showed reduced atrophy compared to placebo-treated EAE mice. (C) Placebo-treated EAE mice demonstrated loss of YFP$^+$ cortical layer V pyramidal neurons in the cerebral cortex compared to healthy controls. This loss was prevented by estriol treatment. (D) Representative 10× images of YFP$^+$ neurons in the cerebral cortex are shown for each treatment group. (E) Representative 63× images of YFP$^+$ dendritic spines on the apical dendrites of cortical layer V neurons in the cerebral cortex for each treatment group. (F) Reduced dendritic spine density was observed during EAE and was prevented with estriol treatment. (G) Estriol treatment protected against loss of synapses in mice with EAE, indicated by the preservation of PSD95 percentage area compared to placebo-treated EAE mice. (H) Representative 10× maximum intensity projection images of axonal damage (ovoids) and axonal transection (end bulbs) in the spinal cord are shown for each treatment group. Quantification of the percentage of axons with ovoids (I) and the percentage of axons with end bulbs (J) reveals preservation of axonal integrity with estriol treatment during EAE. For all box and whisker plots, the center line represents the median, the limits of the box represent the interquartile range (25th-75th percentile), and the whiskers represent 1.5 times the interquartile range. The raw data are overlaid on the box and whisker plot. *$P < .05$, **$P < .01$; Mann-Whitney U test, corrected for false discovery rate. EAE, experimental autoimmune encephalomyelitis; MRI, magnetic resonance imaging; PSD95, postsynaptic density protein 95; YFP, yellow fluorescent protein.

significantly different from healthy controls. In a complementary approach, we examined PSD95 staining, a marker for postsynaptic density, in the cerebral cortex. There was a reduction in PSD95 synaptic staining in placebo-treated EAE mice versus healthy controls ($P = .0080$), whereas estriol-treated EAE mice exhibited more PSD95 staining compared with placebo-treated EAE mice ($P = .019$) (Fig. 2G). Together, these findings demonstrate that estriol treatment after disease onset can restore neuronal and synaptic integrity in the cerebral cortex.

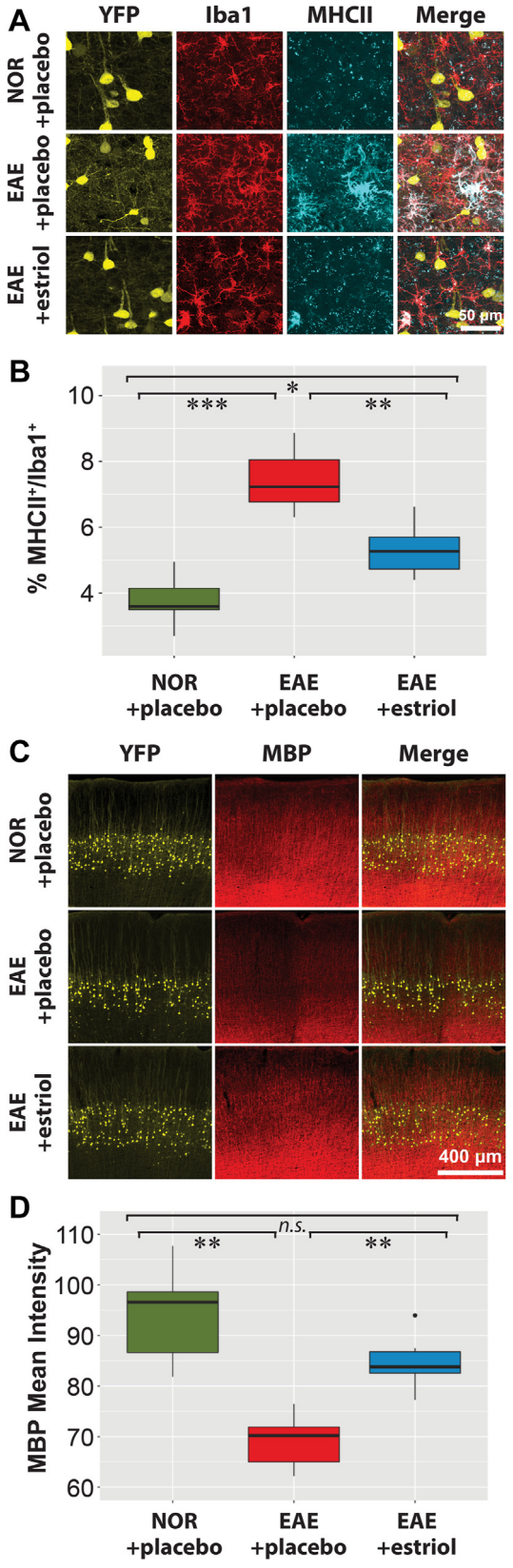

Figure 3.
Estriol treatment reduces microglial activation and preserves myelin in the cerebral cortex during EAE. Thy1-YFP$^+$ mice with EAE treated with placebo (EAE + placebo, red, n = 6), with EAE and treated with estriol (EAE + estriol, blue, n = 7), and healthy controls treated with placebo (NOR + placebo, green, n = 7) were sacrificed 45 days after disease induction for pathological analysis. (A) Representative 40× images of microglial activation in the cerebral cortex for each treatment group. (B) Increased microglial activation in the cerebral cortex was observed during EAE compared with healthy controls. This was reduced with estriol treatment. (C) Representative images of MBP staining in the cerebral cortex are shown for each treatment group. (D) Quantifying MBP mean intensity demonstrates preservation of myelin in the cerebral cortex in estriol-treated compared to placebo-treated EAE mice. For all box and whisker plots, the center line represents the median, the limits of the box represent the interquartile range (25th-75th percentile), and the whiskers represent 1.5 times the interquartile range. The raw data are overlaid on the box and whisker plot. *$P < .05$, **$P < .01$; Mann-Whitney U test, corrected for false discovery rate. EAE, experimental autoimmune encephalomyelitis; Iba1, ionized calcium-binding adapter molecule 1; MBP, myelin basic protein; MHCII, major histocompatibility complex II; YFP, yellow fluorescent protein.

We previously identified a close relationship between axonal damage in the spinal cord and GM volume loss in the cerebral cortex during EAE,[23] and others have suggested that axonal damage plays a key role in GM volume loss.[38,39] Thus, we performed CLARITY on spinal cords and quantified axonal damage (ovoids) and axonal transection (end bulbs) along YFP$^+$ axons. Increased axonal damage and transection were observed in placebo-treated EAE mice compared to healthy controls ($P = .0062$ and $P = .0057$, respectively) (Fig. 2H-J). Importantly, estriol treatment reduced axonal damage and axonal transection compared to placebo-treated EAE mice ($P = .019$ and $P = .0080$, respectively) (Fig. 2H-J).

Estriol Treatment Abrogates Microglial Activation and Preserves Myelin in the Cerebral Cortex in Experimental Autoimmune Encephalomyelitis

Unlike white matter lesions in spinal cords of EAE mice, cerebral cortex GM does not have immune infiltrates from blood. However, resident immune cells, namely microglia, become activated during EAE and can play a role in demyelination[40] and synaptic loss.[36] Thus, we investigated how estriol treatment altered microglial pathology in the cerebral cortex during EAE (Fig. 2A). As expected, microglial activation was increased in the cerebral cortices of placebo-treated EAE mice compared to healthy controls ($P = .0047$) (Fig. 3A, B). Interestingly, estriol treatment in EAE reduced microglial activation in the cerebral cortex compared to placebo-treated EAE mice ($P = .0062$) (Fig. 3A, B). We then quantified myelin integrity and observed a 26.4% reduction in myelin in the cerebral cortex in placebo-treated EAE mice compared to healthy controls ($P = .0047$) (Fig. 3C, D). Estriol treatment in EAE mice preserved myelin in the cerebral cortex compared to placebo-treated EAE mice ($P = .0047$) (Fig. 3C, D).

Together, these data show that therapeutic estriol treatment ameliorates the pathologies within the cerebral cortex previously correlated with GM atrophy in EAE[23] and documented to occur in MS,[36,39] including synaptic and neuronal loss, microglial activation, and demyelination.

Estriol Treatment Disrupts the Neuropathologic Network in Experimental Autoimmune Encephalomyelitis

To understand how estriol treatment affects the relationship between cortical GM atrophy by MRI and cerebral cortex neuropathologies, we performed regression analyses on these measures. When we examined correlations between placebo-treated EAE and healthy control mice, we observed strong relationships between cortical volume loss by MRI and cortical pathologies by

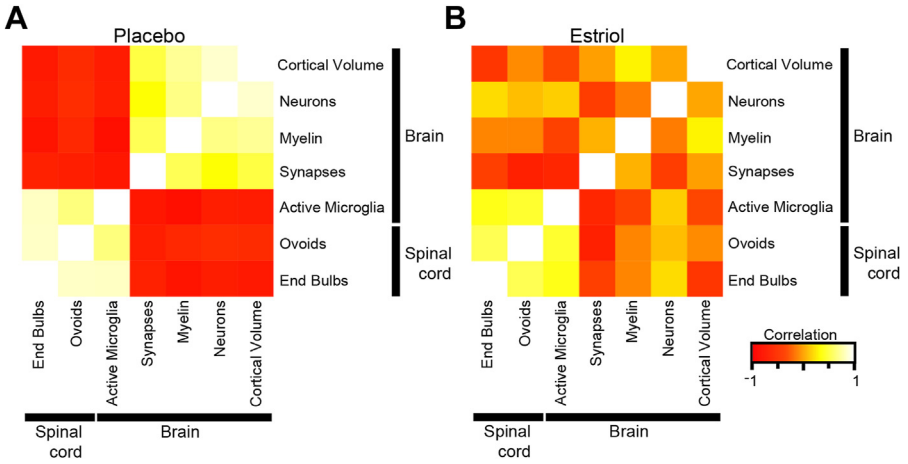

Figure 4.
Estriol treatment disrupts the neuropathologic network in EAE. Heat maps demonstrate Pearson correlations of neuropathologies measured in the cerebral cortex of the same mice in Figures 1 to 3. (A) A heat map of correlations between placebo-treated EAE mice (n = 7) and placebo-treated healthy controls (n = 6) shows a network of significant correlations between neuropathologies in the cerebral cortex, indicating a robust network of associated neuropathologies, a neuropathologic network. (B) Conversely, a heat map in estriol-treated EAE mice (n = 7) and the placebo-treated healthy controls (n = 6) reveals that this neuropathologic network is dramatically disrupted. EAE, experimental autoimmune encephalomyelitis.

immunohistochemistry (Fig. 4A), consistent with previous observations in untreated EAE.[23] Specifically, placebo-treated EAE mice had lower cortical GM volume, fewer cortical layer V neurons, less cortical myelin basic protein (MBP) intensity, and more microglial activation in the cerebral cortex, representing a neuropathologic network. In marked contrast, an examination of correlations between estriol-treated EAE and the same healthy control mice did not show any meaningful correlations between cortical volume loss and cortical layer V neurons, decreased MBP intensity, or microglial activation, representing a disruption of this neuropathologic network (Fig. 4B).

Estriol Treatment Induces Remyelinating Oligodendrocytes and Expression of Cholesterol Synthesis Proteins in the Cerebral Cortex

Because estriol binds principally to estrogen receptor beta (ERβ) over estrogen receptor alpha (ERα),[41,42] and ERβ ligation has been shown to induce remyelination in white matter,[33] we hypothesized that estriol treatment may induce newly remyelinating oligodendrocytes in the cerebral cortex in EAE (Fig. 2A). However, sparing of myelin with therapeutic estriol treatment (Fig. 3C, D) could be due to either induction of remyelination or a decrease in demyelination. We distinguished between these 2 possibilities with the Cspg4-CreERT2/Mapt-mGFP mouse line which provides an ideal model to make this differentiation since these mice express GFP in newly formed remyelinating oligodendrocytes.[31] We induced EAE in Cspg4-CreERT2/Mapt-mGFP mice and treated them with either estriol or placebo (Fig. 5A). The cerebral cortex of estriol-treated EAE mice demonstrated increased GFP percentage area compared to placebo-treated EAE mice ($P = .023$) (Fig. 5B, E). This demonstrated that estriol treatment given after the onset of disease can induce remyelinating oligodendrocytes in the cerebral cortex during EAE.

To investigate a potential mechanism for estriol-induced remyelinating oligodendrocytes in the cerebral cortex, we then examined the expression of cholesterol synthesis proteins in oligodendrocytes (Fig. 2A). Previous results demonstrated an upregulation of cholesterol synthesis genes during remyelination in the corpus callosum during normal diet in the cuprizone model of MS.[32] Here, we found that 2 cholesterol synthesis proteins, hydroxymethylglutaryl-CoA synthase 1 (HMGCS1) and farnesyl diphosphate synthase (FDPS), were increased in expression in cerebral cortex oligodendrocytes in estriol-treated EAE mice compared to placebo-treated EAE mice ($P = .023$ and $P = .026$, respectively) (Fig. 5C, D, F). Together this demonstrated that treatment with an estrogen of pregnancy induced the expression of cholesterol synthesis proteins in oligodendrocytes, increased the number of remyelinating oligodendrocytes, and restored myelin in the cerebral cortex.

ERβ Ligand Treatment is Neuroprotective in the Cerebral Cortex

Estriol is a naturally occurring estrogen that binds preferentially to ERβ and weakly to ERα, whereas selective estrogen receptor modifiers are examples of therapeutic development focused on creating synthetic estrogen receptor ligands to maximize efficacy and minimize toxicity. ERα ligand treatment has previously been shown to mitigate EAE onset and reduce EAE clinical scores in both the acute and subsequent chronic phases of disease.[43-47] In contrast, ERβ ligand-treated mice were shown to be no different as compared to vehicle-treated mice with respect to EAE onset or acute disease course until approximately day 25. Thereafter, scores diverged, whereby ERβ ligand-treated mice demonstrated significant improvement in chronic EAE scores as compared to vehicle-treated mice from day 30 to 50.[33,45,48-51] Synthetic ER ligands have also been previously investigated in the cuprizone demyelination model, where they induced remyelination in the white matter of the spinal cord and corpus callosum and improved axonal conduction.[32,33,48,52] However, ERβ ligands have never been tested for their ability to induce neuroprotection in the cerebral cortex. Here, we treated EAE mice with ERβ ligand and evaluated myelin, microglial activation, and synapses at day 25 since this was when the disease courses between the ERβ ligand- and placebo-treated EAE mice began to diverge. We observed preservation of myelin, a decrease in microglial activation, and preservation of synapses in the cerebral cortex (Fig. 2A) in ERβ ligand-treated compared to placebo-treated

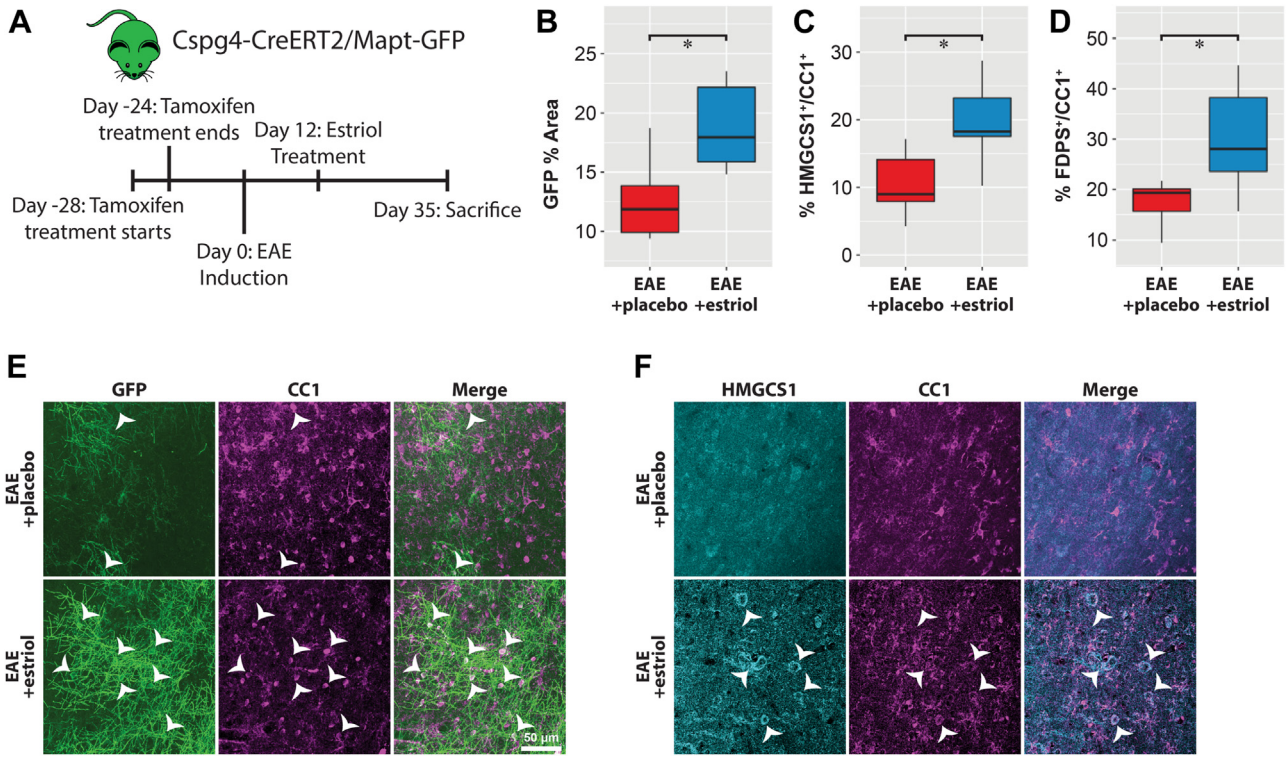

Figure 5.
Therapeutic estriol treatment promotes remyelination and expression of cholesterol synthesis proteins in the cerebral cortex during EAE. (A) Experiment schematic. Tamoxifen was given to Cspg4-CreERT2/Mapt-mGFP mice for 5 consecutive days, 4 weeks prior to EAE induction. Mice were treated either with estriol (EAE + estriol, blue, n = 6) or placebo (EAE + placebo, red, n = 6) 12 days after EAE induction. Animals were sacrificed 35 days after disease induction. (B) Estriol treatment increases GFP expression in the cerebral cortex compared to placebo-treated EAE mice. (C) Estriol treatment increases the expression of the cholesterol synthesis protein HMGCS1 in $CC1^+$ oligodendrocytes in the cerebral cortex compared to placebo-treated mice with EAE. (D) Estriol treatment increases the expression of the cholesterol synthesis protein FDPS in $CC1^+$ oligodendrocytes in the cerebral cortex compared to placebo-treated mice with EAE. (E) Representative 40× images of GFP expression in the cerebral cortex are shown for each treatment group. The arrowheads identify mature, remyelinating oligodendrocytes expressing GFP, colocalized with CC1, a marker for mature oligodendrocytes. (F) Representative 40× images of HMGCS1 expression in the cerebral cortex are shown for each treatment group. The arrowheads identify HMGCS1 expression colocalized with CC1. For all box and whisker plots, the center line represents the median, the limits of the box represent the interquartile range (25th-75th percentile), and the whiskers represent 1.5 times the interquartile range. The raw data are overlaid on the box and whisker plot. *$P < .05$; Mann-Whitney U test, corrected for false discovery rate. CC1, anti-adenomatous polyposis coli clone; EAE, experimental autoimmune encephalomyelitis; FDPS, farnesyl diphosphate synthase; GFP, green fluorescent protein; HMGSC1, hydroxymethylglutaryl-CoA synthase 1.

EAE mice (Fig. 6A-D). Once again, to distinguish between the 2 possibilities (i.e., treatment-mediated induction of remyelinating oligodendrocytes versus reduction in demyelination) we used the Cspg4-CreERT2/Mapt-mGFP mouse line that expresses GFP in newly formed remyelinating oligodendrocytes.[31] We induced EAE in these mice and observed increased myelin and GFP expression in the cerebral cortex compared to placebo-treated EAE mice (Fig. 6E-H). Thus, treatment with the selective ERβ ligand reduced microglial activation, preserved synapses, increased myelin staining, and induced the formation of remyelinating oligodendrocytes in the cerebral cortex during EAE.

Discussion

GM atrophy, as measured by MRI, is associated with disability progression in MS, yet there are currently no direct neuroprotective treatments designed to prevent it. Recent evidence suggested that estriol treatment can prevent cortical GM atrophy in patients with MS,[18-20,22] but neuroprotective mechanisms within the cerebral cortex remain unclear. Here, we utilized the chronic EAE model of MS to investigate estriol-mediated neuroprotection in the cerebral cortex. Estriol treatment to induce serum estriol levels consistent with pregnancy in mice[27] was initiated after disease onset, which mitigated cerebral cortex atrophy as measured by in vivo MRI. This was also observed when estriol treatment was initiated before EAE induction (Supplementary Fig. S1). This estriol-mediated sparing of cerebral cortex atrophy was accompanied by preservation of cortical layer V neurons and their apical dendrites, as well as synapses in the cerebral cortex. Estriol treatment also induced expression of cholesterol synthesis proteins in oligodendrocytes, increased the number of remyelinating oligodendrocytes, and restored myelin within the cerebral cortex. Since estriol is a natural estrogen that binds primarily to ERβ, we demonstrated that treatment with a highly selective synthetic ERβ ligand also induced this neuroprotection in the cerebral cortex during EAE.

Oligodendrocytes and microglia have been identified as the cellular targets for ERβ ligand treatment-mediated neuroprotection in white matter of the spinal cord during EAE.[33,32,53] Recently, RNA sequencing analyses in remyelinating oligodendrocytes of the corpus callosum in the cuprizone model showed an upregulation of cholesterol synthesis genes, and chromatin immunoprecipitation (ChIP) assays showed estrogen receptor binding to estrogen response elements of cholesterol synthesis

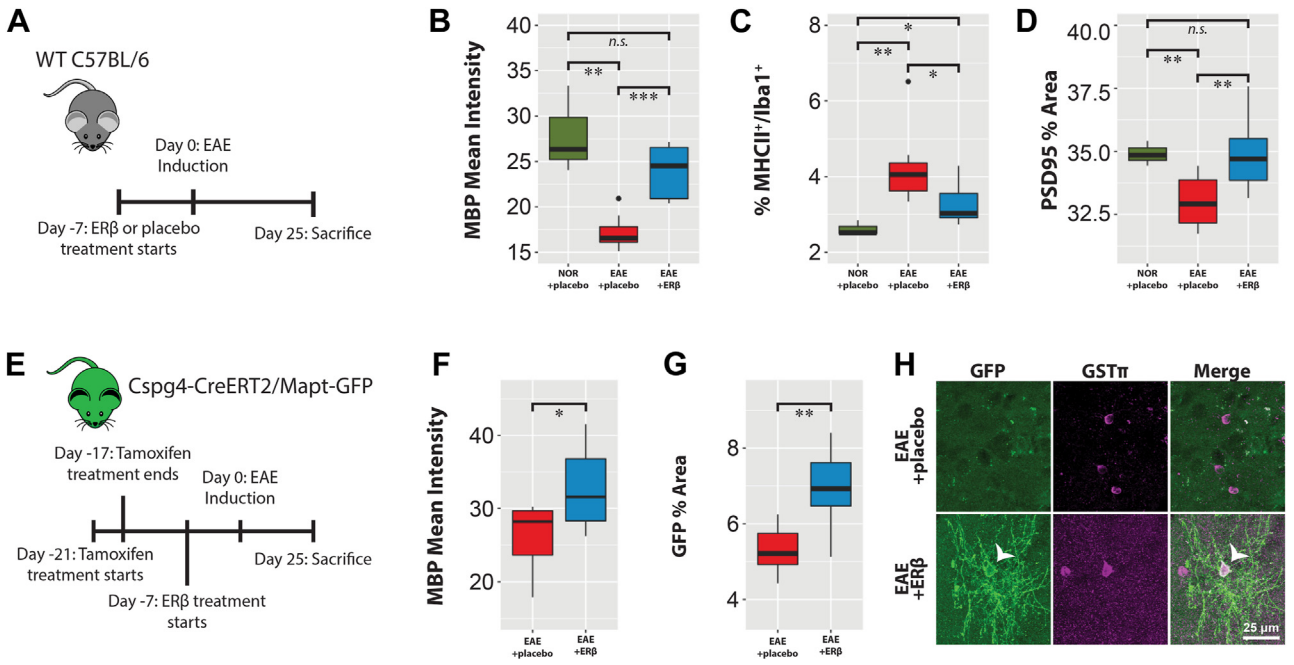

Figure 6.

ERβ ligand treatment is neuroprotective and induces newly remyelinating oligodendrocytes in the cerebral cortex during EAE. (A) Experiment schematic. Wild-type C57BL/6 mice were treated either with ERβ ligand (EAE + ERβ, blue, n = 10) or placebo injections (EAE + placebo, red, n = 8) one week prior to EAE induction. Animals were sacrificed alongside placebo-treated healthy controls (NOR + placebo, green, n = 3–5) 25 days after disease induction. (B) ERβ ligand treatment preserves MBP intensity in the cerebral cortex compared with placebo-treated EAE mice. (C) ERβ ligand treatment reduces microglial activation in the cerebral cortex compared with placebo-treated EAE mice. (D) ERβ ligand treatment preserves PSD95-stained synapses compared with placebo-treated EAE mice. (E) Experiment schematic. Tamoxifen was given to Cspg4-CreERT2/Mapt-mGFP mice for 5 consecutive days, 2 weeks prior to ERβ ligand treatment. Mice were treated either with ERβ ligand (EAE + ERβ, blue, n = 10) or placebo injections (EAE + placebo, red, n = 9) one week prior to EAE induction. Animals were sacrificed 25 days after disease induction. (F) ERβ ligand treatment preserves MBP intensity in the cerebral cortex compared with placebo-treated EAE mice. (G) ERβ ligand treatment increases GFP expression in the cerebral cortex compared to placebo-treated EAE mice. (H) Representative 40× images of GFP expression in the cerebral cortex are shown for each treatment group. The arrowhead identifies a mature, remyelinating oligodendrocyte expressing GFP, colocalized with GSTΠ, a marker for mature oligodendrocytes, in an ERβ ligand-treated mouse. For all box and whisker plots, the center line represents the median, the limits of the box represent the interquartile range (25th-75th percentile), and the whiskers represent 1.5 times the interquartile range. The raw data are overlaid on the box and whisker plot. *$P < .05$, **$P < .01$; Mann-Whitney U test, corrected for false discovery rate. EAE, experimental autoimmune encephalomyelitis; ERβ, estrogen receptor beta; GFP, green fluorescent protein; GSTΠ, glutathione S-transferase-Π; Iba1, ionized calcium-binding adapter molecule 1; MBP, myelin basic protein; MHCII, major histocompatibility complex II; PSD95, postsynaptic density protein 95.

genes.[33,32] This is intriguing when considering developmental myelination. During fetal and neonatal development, cholesterol synthesis in oligodendrocytes supports myelin formation.[54,55] During pregnancy, estriol is made by the fetoplacental unit, with the highest maternal serum estriol levels in the third trimester when fetal myelination is most robust. Our new data in this context suggests that estriol treatment in adults recapitulates developmental myelination by binding to ERβ on OPCs to upregulate cholesterol synthesis genes and enhance remyelination in the cerebral cortex. Remyelination is thought to provide trophic support to axons, thereby preserving axons, neurons, and synapses. These pathologies correlated with cortical GM atrophy in EAE and were abrogated by estriol treatment in EAE.

We chose to use the chronic EAE model due to its pathological similarities to MS, namely demyelination in the presence of inflammation.[26] In both MS and EAE, it is thought that the inflammatory microenvironment impedes remyelination in spinal cord white matter.[31,33] Increased microglial activation has also been observed in the hippocampus and cerebral cortex during MS and EAE.[23,29] Activated microglia produce nitric oxide and reactive oxygen species that induce axonal damage and oligodendrocyte apoptosis.[38,56] Activated microglia may also be involved in complement-mediated synaptic loss in MS and EAE.[36,37] Interestingly, ERβ ligand treatment was previously shown to act directly on CD11c$^+$ microglia/macrophages in spinal cord white matter to reduce the M1 phenotype and ameliorate clinical EAE severity scores.[33] Here, we found for the first time that both estriol and ERβ ligand treatment reduced microglia activation in the cerebral cortex to not only induce remyelination but also to protect synapses.

Interestingly, pretreatment with estriol was shown to ameliorate clinical disease in EAE in both male and female mice.[28] ERβ is expressed extensively throughout the brain, particularly in the cerebral cortex in female mice.[57] Although there are reports of localized sex differences in ERβ expression in the brains of rats, the overall expression is high throughout the brain in both sexes.[58] Therefore, we expect that estriol-treated male mice would exhibit similar effects to estriol-treated female mice, decreased cerebral cortex atrophy, more remyelinating oligodendrocytes, and increased myelin in the cerebral cortex. We have not performed these experiments since, as a potential therapy for MS, estriol treatment may lead to unwanted side effects in male patients. However, we have previously tailored sex hormone treatment for males using testosterone.[59-62] Testosterone treatment in men has an established safety record and is converted to estradiol by aromatase,[63,64] so either would confer neuroprotection. In fact, male MS patients treated with testosterone did not exhibit GM atrophy but demonstrated an increase in GM volume in the right frontal cortex after 12 months of treatment.[60]

In a previous estriol treatment trial in MS, a direct neuroprotective effect of estriol treatment was suggested by the reduction in cerebral cortex atrophy in patients with MS who were gadolinium-enhancing lesion negative.[18] Also, estriol-treated patients showed significant improvement in cognitive testing compared with placebo-treated patients, and higher estriol levels correlated with better cognitive performance.[18] Furthermore, a correlation was observed between improvement on the paced auditory serial addition test and sparing of cerebral cortex atrophy in all subjects.[18] Voxel-based morphometry revealed regional cortical GM sparing in the estriol group compared with the placebo group. This included a cluster of GM sparing in the medial frontal cortex,[22] a region known to be involved in problem-solving[65] and attention[66] and to be activated on functional MRI during arithmetic tasks,[67,68] which correlated with improved paced auditory serial addition test scores. Unfortunately, imaging acquisition did not permit evaluation of remyelination in previous estriol trials.[17,18] Findings herein warrant investigation of estriol treatment to induce remyelination as measured by magnetization transfer ratio,[69,70] myelin water fraction,[71,72] positron emission tomography,[73,74] or a multispectral approach[75] in the cerebral cortex to determine the relationship between cortical remyelination and cortical GM atrophy sparing.

Estriol is an attractive therapeutic option because it preferentially binds to ERβ over ERα. In contrast to estradiol, which preferentially binds ERα and has been associated with an increase in breast cancer risk and other off-target side effects,[76] these toxicities have not been observed during estriol treatment, which is thought to be due to its preferential binding to ERβ.[18,77] Indeed, adverse effects on breast, gynecologic outcomes, or clotting were not observed in MS clinical trials of estriol treatment[17,18] and have not been reported in postmarketing experience during estriol use for decades in Europe and Asia as a hormone replacement therapy to treat hot flashes and other menopausal symptoms (prevention of urinary tract infections, vaginal dryness, and osteoporosis).[77-88] Future clinical trials of estriol treatment as a neuroprotective treatment for MS are warranted based on preclinical and clinical data on efficacy as well as its safety track record, together representing a risk-benefit ratio within the realm of other disease-modifying treatments approved and in development for MS.

Author Contributions

C.E.M., R.R.V., and A.M.G. designed the study. C.E.M., A.A.P.R., V.F., N.I., J.L.G., K.H.N., and M.R.O. induced, monitored, and treated mice with EAE. C.E.M. and A.M.G. collected MRIs. C.E.M. analyzed the MRIs. C.E.M. performed CLARITY. C.E.M., A.W.S., A.A.P., J.L.G., P.D.H., Q.N., and K.H.N. performed the immunohistochemistry. C.E.M., A.W.S., A.A.P.R., J.L.G., P.D.H., Q.N., and K.H.N. performed the microscopy. C.E.M., A.W.S., A.A.P.R., J.L.G., P.D.H., Q.N., and K.H.N. analyzed the images. C.E.M., Y.I., and P.S. performed the statistical analyses. C.E.M., R.R.V., and A.M.G. wrote the manuscript. C.E.M. and Y.I. generated the figures. A.M.G. supervised the research. C.E.M., R.R.V., and A.M.G. received funding. All authors read and approved the final manuscript.

Data Availability

Magnetic resonance, CLARITY, and immunohistochemistry images from this study are available from the authors upon request.

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Declaration of Competing Interest

R.R.V. is an inventor of UCLA patents pertaining to estriol and ERβ ligand treatments. The authors had full access to all the data in this study and take complete responsibility for the integrity of the data and the accuracy of the data analysis.

Ethics Approval and Consent to Participate

All procedures were performed in accordance with the guidelines of the National Institutes of Health and the Chancellor's Animal Research Committee of the University of California, Los Angeles.

Supplementary Material

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