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Neuroprotection in cerebral cortex induced by the pregnancy hormone estriol

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Abstract

In multiple sclerosis (MS), demyelination occurs in 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 cerebral cortex in the preclinical model of MS, experimental autoimmune encephalomyelitis (EAE). Estriol treatment initiated after disease onset decreased cerebral cortex atrophy. Neuropathology of 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 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 cerebral cortex.

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Author Contribution Statement

CEM, RRV, and AMG designed the study; CEM, AAPR, VF, NI, JLG, KHN, and MRO induced, monitored, and treated mice with EAE; CEM and AMG collected MRI;CEM analyzed MRI; CEM performed CLARITY; CEM, AWS, AAP, JLG, PDH, QN, and KHN performed immunohistochemistry; CEM, AWS, AAPR, JLG, PDH, QN, and KHN performed microscopy; CEM, AWS, AAPR, JLG, PDH, QN, and KHN analyzed images; CEM, YI, and PS performed statistical analyses; CEM, RRV, and AMG wrote the manuscript; CEM and YI generated figures; AMG supervised the research, CEM, RRV, and AMG received funding. All authors read and approved the final manuscript.

Ethics Approval

All procedures were done 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.

Conflict of Interest Statement

Dr. Rhonda Voskuhl is an inventor on UCLA patents pertaining to estriol and ERB 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.

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 than either white matter atrophy or white matter lesion load at predicting motor disability[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 (DMTs) have robust immunomodulatory effects in the peripheral immune system and 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. There are decreased relapses 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 a time when myelination is beginning and synaptic plasticity is occurring 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 gray matter atrophy, particularly in those with no enhancing lesions. Also, reduced cerebral cortex atrophy correlated with improvement in cognitive testing performance[18–20,22]. Determining mechanisms underlying neuroprotective effects of estriol treatment in 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] and as reviewed[26]. Estriol treatment in EAE induced a beneficial immunomodulatory effect in the peripheral immune system, reduced spinal cord pathology, and ameliorated walking disability[27,28]. Further, estriol has been shown to preserve hippocampal CA1 volume loss and prevent decreases in synaptic number and transmission in the hippocampus of mice with EAE[29]. Here, we determined whether estriol treatment given therapeutically 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 cerebral cortex during EAE in C57BL/6 mice as well as in informative mouse lines, namely Thy1-YFP$^+$ mice that express 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].

Results

Estriol treatment after disease onset spares cortical atrophy in EAE

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 then 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 ($p = 0.0047$) (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 ($p = 0.012$) (Fig. 2a, b).

Estriol-treatment reverses neurodegeneration in the cerebral cortex

Next, we investigated cellular mechanisms underlying the estriol-mediated protection from cortical atrophy. In the same mice used for *in vivo* MRI analysis, we quantified cortical layer V pyramidal neurons in the same region as was evaluated by MRI (Fig. 2a). The mice used were Thy1-YFP$^+$ and expressed yellow fluorescent protein 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 ($p = 0.0047$) (Fig. 2c, d). Interestingly, cortical layer V neurons were preserved in estriol-treated EAE mice compared to placebo-treated EAE mice ($p = 0.0047$) (Fig. 2c, d) and indeed were not significantly different than healthy controls. We next examined synaptic integrity in cerebral cortex, since synapse loss is known to occur in MS[32] and EAE[29,33]. Dendritic spine density on the apical dendrites of cortical layer V pyramidal neurons showed a reduction in placebo-treated EAE mice versus healthy controls ($p = 0.0080$) (Fig. 2e, f). In contrast, estriol-treated EAE mice demonstrated sparing of spine density compared to placebo-treated EAE mice ($p = 0.044$) (Fig. 2e, f) and were not 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 PSD-95 synaptic staining in placebo-treated EAE mice versus healthy controls ($p = 0.0080$), whereas we observed that estriol-treated EAE mice exhibited more PSD95 staining compared to placebo-treated EAE mice ($p = 0.019$) (Fig. 2g). Together these findings demonstrate that estriol treatment after disease onset can restore neuronal and synaptic integrity in cerebral cortex.

We previously identified an intimate 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[34,35]. Thus, we performed CLARITY on spinal cords and quantified axonal damage (ovoids) and axonal transection (end bulbs) along YFP^+ axons. Increased axonal damage and axonal transection were observed in placebo-treated EAE mice compared to healthy controls ($p = 0.0062$ and $p = 0.0057$, respectively) (Fig. 2h–j). Importantly, estriol treatment reduced axonal damage and axonal transection when compared to placebo-treated EAE mice ($p = 0.019$ and $p = 0.0080$, respectively) (Fig. 2h–j).

Estriol treatment abrogates microglial activation and preserves myelin in cerebral cortex in EAE

Unlike white matter lesions in spinal cords of EAE mice, cerebral cortex gray matter does not have immune infiltrates from blood. However, resident immune cells, namely microglia, become activated during EAE and can play a role in demyelination[36] and synaptic loss[37]. 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 mice with EAE compared to healthy controls ($p = 0.0047$) (Fig. 3a, b). Interestingly, estriol treatment in EAE reduced microglial activation in cerebral cortex compared to placebo-treated EAE mice ($p = 0.0062$) (Fig. 3a, b). We then quantified myelin integrity and observed a 26.4% reduction in myelin in cerebral cortex in placebo-treated EAE mice compared to healthy controls ($p = 0.0047$) (Fig. 3c, d). Estriol treatment in EAE preserved myelin in the cerebral cortex compared to placebo-treated EAE ($p = 0.0047$) (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[32,35], including synaptic and neuronal loss, microglial activation, and demyelination.

Estriol treatment disrupts the neuropathologic network in EAE

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 immunohistochemistry (Fig. 4a), consistent with previous observations in untreated EAE[38]. Specifically, placebo-treated EAE mice had lower cortical GM volume, fewer cortical layer V neurons, and less cortical MBP intensity, as well as 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 cerebral cortex

Since estriol binds principally to estrogen receptor beta (ERβ) over estrogen receptor alpha (ERα)[39,40], and ERβ ligation has been shown to induce remyelination in white matter[41], we hypothesized that estriol treatment may induce newly remyelinating oligodendrocytes in 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 two possibilities. The Cspg4-CreERT2/Mapt-mGFP mouse line provides an ideal model to make this distinction, since these mice express green fluorescent protein in newly formed remyelinating oligodendrocytes[31]. We induced EAE in Cspg4-CreERT2/Mapt-mGFP mice and treated with either estriol or placebo (Fig. 5a). The cerebral cortex of estriol-treated EAE mice demonstrated increased GFP % area compared to placebo-treated EAE mice ($p = 0.023$) (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[42]. Here, we found that two 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 ($p = 0.023$ and $p = 0.026$, 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 cerebral cortex

Estriol is a naturally occurring estrogen that binds preferentially to ERβ and weakly to ERα, whereas selective estrogen receptor modifiers (SERMs) 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 diverge whereby ERβ ligand-treated demonstrated significant improvement in chronic EAE scores as compared to vehicle-treated from day 30 to endpoint day 50[41,45,48–51]. Synthetic ER ligands have also been previously investigated in the cuprizone demyelination model where they induced remyelination in white matter of spinal cord and corpus callosum and improved axonal conduction[41,42,48,52]. 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 EAE mice (Fig. 6a–d). Once again, to distinguish between the two possibilities, 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 both increased myelin and increased 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 directly 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 cerebral cortex remain unclear. Here, we utilized the chronic EAE model of MS to investigate estriol-mediated neuroprotection in 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. 1). 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 cerebral cortex. Since estriol is a natural estrogen that binds primarily to ERβ, we then demonstrated that treatment with a highly selective synthetic ERβ ligand also induced this neuroprotection in cerebral cortex during EAE.

Oligodendrocytes and microglia have been identified as the cellular targets for ERβ-ligand treatment mediated neuroprotection in white matter of spinal cord during EAE[41,42,53]. Recently, RNA sequencing analyses in remyelinating oligodendrocytes of the corpus callosum in the cuprizone model showed an upregulation of cholesterol synthesis genes, and ChIP assays showed estrogen receptor binding to estrogen response elements of cholesterol synthesis genes[41,42]. 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 cerebral cortex. Remyelination is thought to provide trophic support to axons, thereby preserving axons, neurons, and synapses, indeed the very pathologies that 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[31,41]. 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 which induce axonal damage and oligodendrocyte apoptosis[34,56]. Activated microglia may also be involved in complement mediated synaptic loss in MS and EAE[32,33]. 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[41]. 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], and 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 gray matter atrophy, but demonstrated an increase in gray matter 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 MS patients who were gadolinium-enhancing lesion negative[18]. Also, estriol-treated patients showed significant improvement in cognitive testing as compared to placebo-treated, and higher estriol levels correlated with better cognitive performance[18]. Further, a correlation was observed between improvement in PASAT testing and sparing of cerebral cortex atrophy in all subjects[18]. Voxel-based morphometry (VBM) revealed regional cortical GM sparing in the estriol group compared to 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 fMRI during arithmetic tasks[67,68] which correlated with improvement with PASAT 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 (MTR)[69,70], myelin water fraction (MWF)[71,72], positron emission tomography (PET)[73,74], or a multispectral approach[75] in 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, thought 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 post-marketing 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.

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, ME) and bred within our facilities. 20 female Thy1-YFP$^+$ C57BL/6J mice were used in the therapeutic estriol treatment study. This experiment was validated with two independent cohorts of 21 and 15 mice respectively. 24 female wild-type C57BL/6J mice were used in the experiment assessing the effect of ERβ-ligand on cortical pathology. This experiment was validated with an independent cohort of 19 mice. 19 female Cspg4-CreERT2/Mapt-mGFP mice were used in the experiment investigating 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[31,42]. 28 female Thy1-YFP$^+$ C57BL/6J mice were used in the estriol pre-treatment study (Supplementary Fig. 1).

EAE induction and experimental treatment:

EAE was induced as described[23]. EAE was scored a scale of 0–5, as described[41].

In the therapeutic estriol treatment experiment, either a 90-day release pellet of estriol at 5 mg dose or a placebo pellet (Innovative Research of America, Sarasota, FL) was implanted as described[27] after mice showed symptoms of EAE. In the pre-treatment experiment (Supplementary Fig. 1) either an estriol or placebo pellet was similarly implanted one 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 (DPN; Tocris, Minneapolis, MN) was administered as described[41,42]. For Cspg4-CreERT2/Mapt-mGFP transgenic mice, Tamoxifen (Sigma-Aldrich, St. Louis, MO) was administered subcutaneously two weeks prior ERβ-ligand treatment for 5 consecutive days, as described[41,42].

Magnetic resonance imaging:

All animals were scanned *in vivo* at the Ahmanson-Lovelace Brain Mapping Center at UCLA on a 7T Bruker imaging spectrometer (Bruker Instruments, Billerica, MA), as described[23].

Atlas-based morphometry (ABM):

A minimum deformation atlas (MDA) was created using the MR images from all subjects. The cerebral cortex was manually labeled on the MDA using BrainSuite 18a[89] (http://brainsuite.org) as described[24]. The labels were warped out to the individual images and manually corrected by an investigator blind to disease and treatment group (CEM).

Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging-compatible Tissue-hYdrogel:

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

Immunohistochemistry:

Sagittal brain sections from a subset of animals were stained using immunohistochemistry, as described[23]. Tissues were stained for postsynaptic density protein 95 (PSD95; Millipore, Darmstadt, Germany), myelin basic protein (MBP; Aves, Davis, CA), green fluorescent protein (GFP; Abcam, Cambridge, MA), Glutathione S-transferase pi (GSTπ; Enzo Life Sciences, Farmingdale, NY), major histocompatibility complex II (MHC-II; BioLegend, San Diego, CA), ionized calcium-binding adapter molecule 1 (Iba1; Wako, Richmond, VA), anti-adenomatous polyposis coli clone (CC1; Calbiochem, San Diego, CA), Farnesyl diphosphate synthase (FDPS; Invitrogen, Waltham, MA), or hydroxymethylglutaryl-CoA synthase (HMGCS1; Invitrogen, Waltham, MA).

Microscopy:

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

Statistical analyses:

ABM, CLARITY, and immunohistochemistry data were analyzed in R (https://www.r-project.org). Two-group comparisons were conducted using a Mann-Whitney U test (two-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 (FDR)[90] within each experiment. 95% confidence intervals were found by resampling (10,000 bootstraps).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability Statement

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

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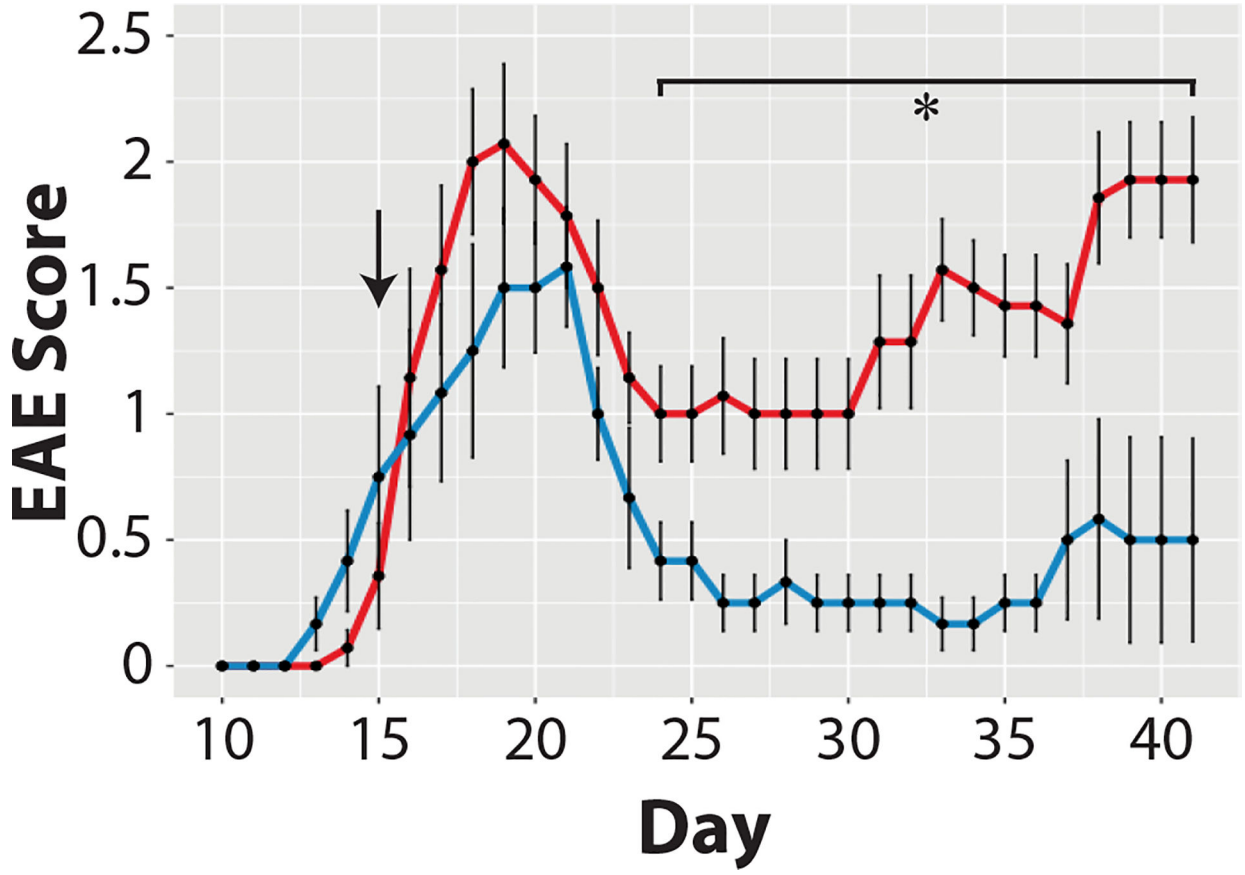

Fig. 1: Therapeutic estriol treatment reduces clinical scores.

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 experimental autoimmune encephalomyelitis (EAE) severity compared to placebo-treated mice 23 days after disease induction. The ameliorative effect continued through 45 days after disease induction when the animals were sacrificed. A one-way ANOVA indicated a significant effect of estriol treatment ($F(1,62) = 22.85$, $p = 0.000011$). The asterisk indicates a significant difference ($p < 0.05$, FDR corrected) in EAE score between placebo-treated and estriol-treated EAE mice.

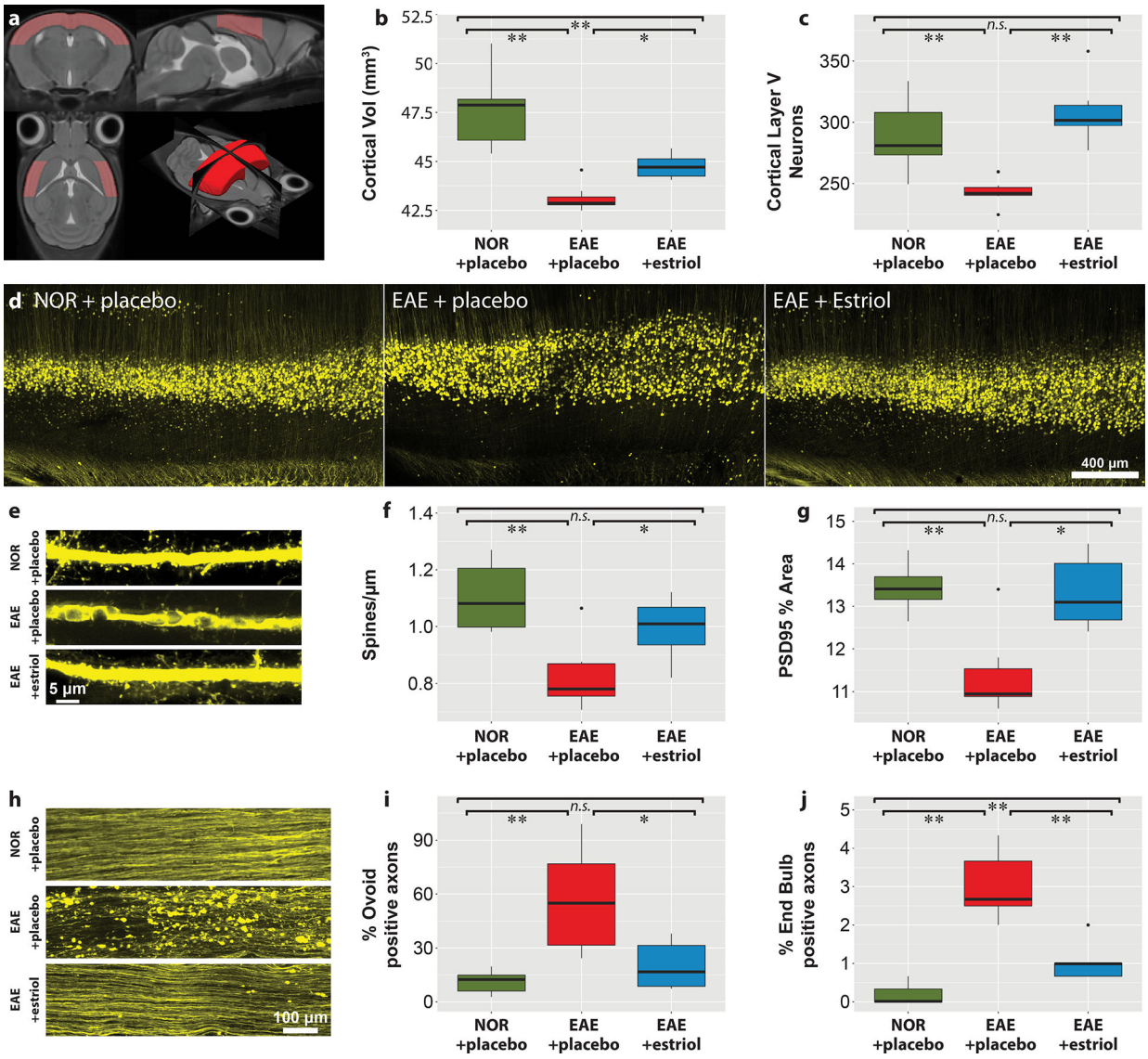

Fig. 2: Estriol treatment after disease onset reduces cortical gray matter atrophy and mitigates underlying neurodegeneration in cerebral cortex during EAE.

In vivo MRI was collected at d45 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 (MDA) was constructed from all the images in the dataset and a cerebral cortex was delineated on the MDA and warped out to the constituent images. **b** Significant atrophy was observed in placebo-treated EAE mice when compared to placebo-treated healthy control mice. Estriol-treated EAE mice showed reduced atrophy when compared to placebo-treated EAE mice. **c** Placebo-treated EAE mice demonstrated loss of YFP$^+$ cortical layer V pyramidal neurons in cerebral cortex compared to healthy controls. This loss was prevented by estriol treatment. **d** Representative 10X images of YFP$^+$ neurons in cerebral cortex are shown for each treatment group. **e** Representative 63X 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 preservation of PSD95 % area compared to placebo-treated mice with EAE. **h** Representative 10X 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 % of axons with ovoids (**i**) and % 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 (25^{th}-75^{th} percentile), and the whiskers represent 1.5 times the interquartile range. The raw data is overlaid on the box and whisker plot. *$p < 0.05$, **$p < 0.01$; Mann-Whitney U test, FDR corrected.

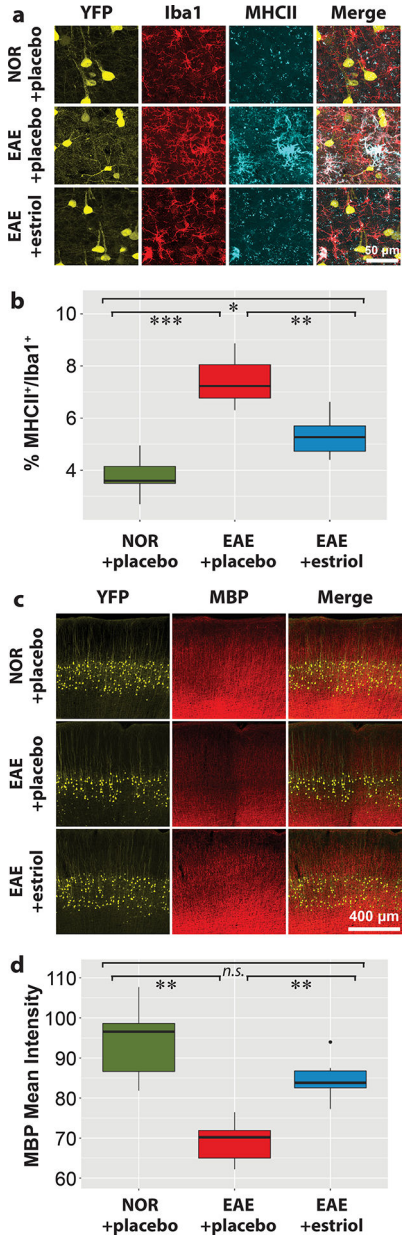

Fig. 3: Estriol treatment reduces microglial activation and preserves myelin in cerebral cortex during EAE.

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 40X images of microglial activation in cerebral cortex for each treatment group. **b** Increased microglial activation in cerebral cortex was observed during EAE as compared to healthy controls. This was reduced with estriol treatment. **c** Representative images of MBP staining in cerebral cortex are shown for each treatment group. **d** Quantification of MBP mean intensity demonstrates preservation of myelin in 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 (25^{th}-75^{th} percentile), and the whiskers represent 1.5 times the interquartile range. The raw data is overlaid on the box and whisker plot. *$p < 0.05$, **$p < 0.01$; Mann-Whitney U test, FDR corrected.

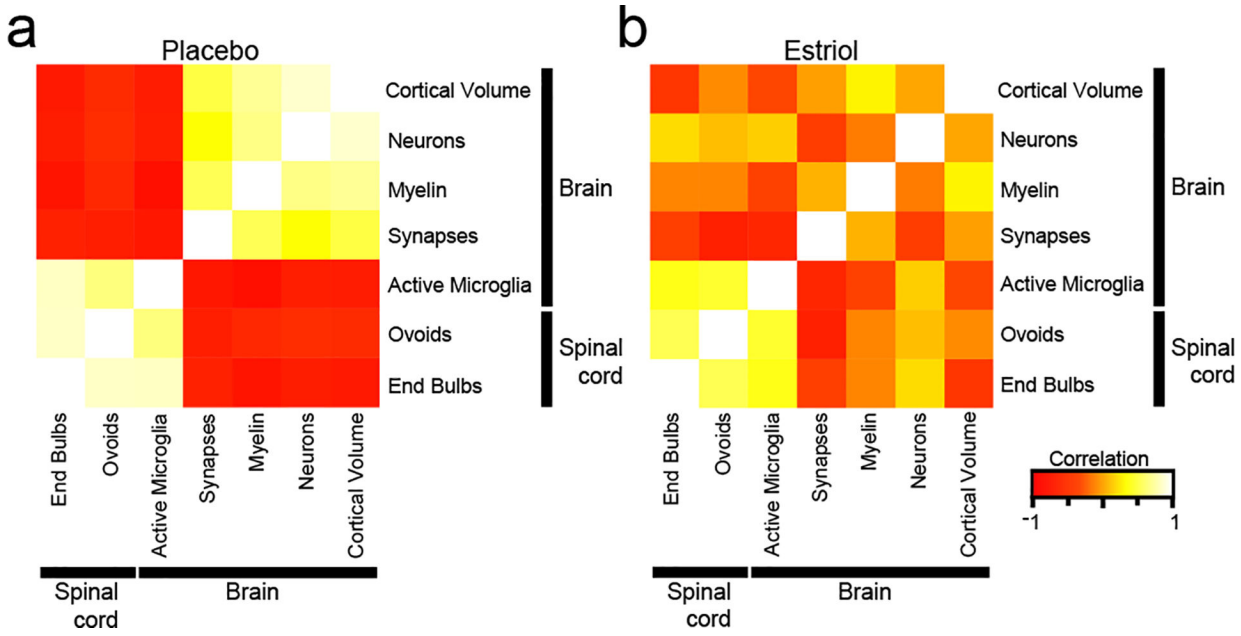

Fig. 4: Estriol treatment disrupts the neuropathologic network in EAE.

Heat maps demonstrate Pearson correlations of neuropathologies measured in the cerebral cortex of the same mice in Figs. 1–3. A heat map of correlations between placebo-treated EAE mice (n = 7) and placebo-treated healthy controls (n = 6) (left) shows a network of significant correlations between neuropathologies in the cerebral cortex, indicating a robust network of associated neuropathologies, a neuropathologic network. Conversely, a heat map in estriol-treated EAE mice (n = 7) and the placebo-treated healthy controls (n = 6) (right) reveals that this ‘neuropathologic network is dramatically disrupted.

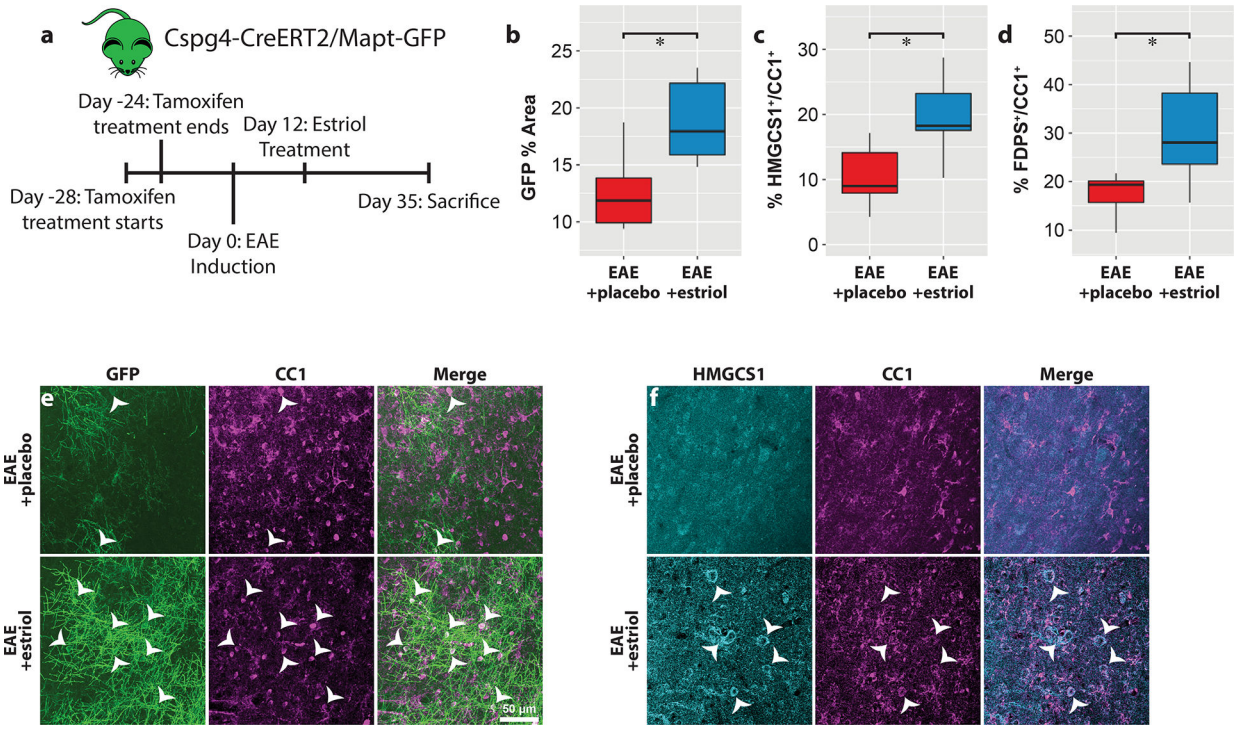

Fig. 5: Therapeutic estriol treatment promotes remyelination and expression of cholesterol synthesis proteins in the cerebral cortex during EAE.

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 at 35 days after disease induction. **b** Estriol treatment increases GFP expression in cerebral cortex compared to placebo-treated EAE mice. **c** Estriol treatment increases the expression of the cholesterol synthesis protein HMGCS1 in $CC1^+$ oligodendrocytes in 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 cerebral cortex compared to placebo-treated mice with EAE. **e** Representative 40X images of GFP expression in 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 40X 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 (25^{th}-75^{th} percentile), and the whiskers represent 1.5 times the interquartile range. The raw data is overlaid on the box and whisker plot. *$p < 0.05$, Mann-Whitney U test, FDR corrected.

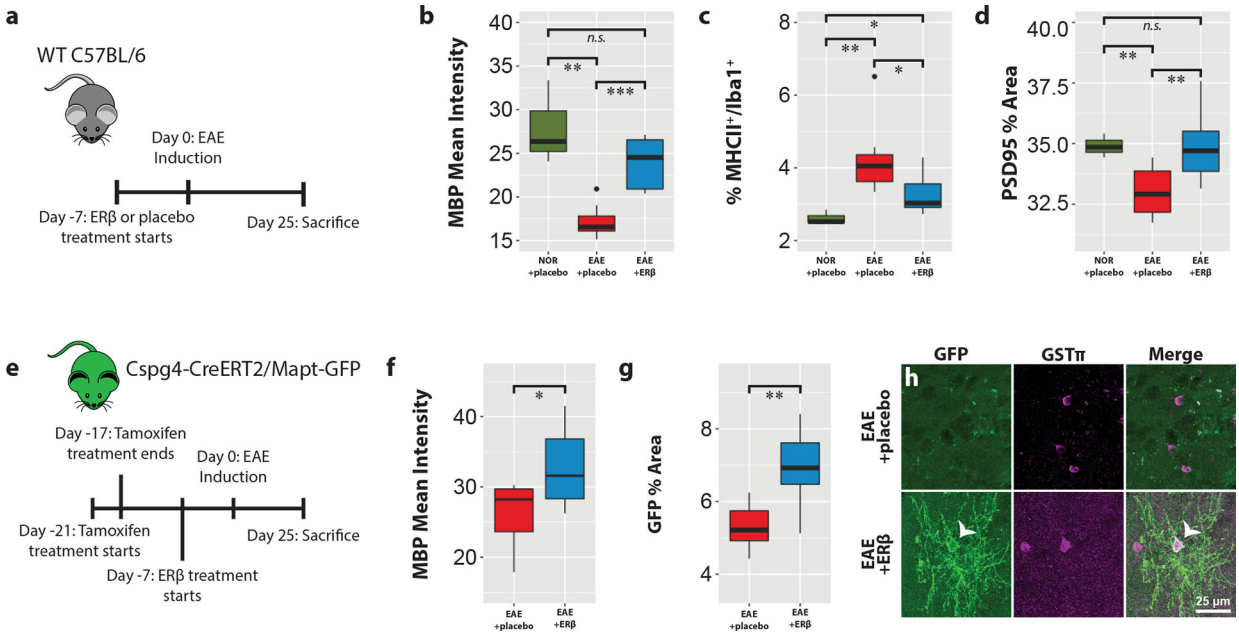

Fig. 6: ERβ-ligand treatment is neuroprotective and induced newly remyelinating oligodendrocytes in the cerebral cortex during EAE.

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) at 25 days after disease induction. **b** ERβ-ligand treatment preserves MBP intensity in cerebral cortex compared to placebo-treated EAE mice. **c** ERβ-ligand treatment reduces microglial activation in cerebral cortex compared to placebo-treated EAE mice. **d** ERβ-ligand treatment preserves PSD95-stained synapses compared to 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 at 25 days after disease induction. **f** ERβ-ligand treatment preserves MBP intensity in cerebral cortex compared to placebo-treated EAE mice. **g** ERβ-ligand treatment increases GFP expression in cerebral cortex compared to placebo-treated EAE mice. **h** Representative 40X 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 GST*Π*, 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 (25^{th}-75^{th} percentile), and the whiskers represent 1.5 times the interquartile range. The raw data is overlaid on the box and whisker plot. *p < 0.05, **p < 0.01; Mann-Whitney U test, FDR corrected.