Estriol preserves synaptic transmission in the hippocampus during autoimmune demyelinating disease

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Cognitive deficits occur in over half of multiple sclerosis patients, with hippocampal-dependent learning and memory commonly impaired. Data from *in vivo* MRI and post-mortem studies in MS indicate that the hippocampus is targeted. However, the relationship between structural pathology and dysfunction of the hippocampus in MS remains unclear. Hippocampal neuropathology also occurs in experimental autoimmune encephalomyelitis (EAE), the most commonly used animal model of MS. Although estrogen treatment of EAE has been shown to be anti-inflammatory and neuro-protective in the spinal cord, it is unknown if estrogen treatment may prevent hippocampal pathology and dysfunction. In the current study we examined excitatory synaptic transmission during EAE and focused on pathological changes in synaptic protein complexes known to orchestrate functional synaptic transmission in the hippocampus. We then determined if estriol, a candidate hormone treatment, was capable of preventing functional changes in synaptic transmission and corresponding hippocampal synaptic pathology. Electrophysiological studies revealed altered excitatory synaptic transmission and paired-pulse facilitation (PPF) during EAE. Neuropathological experiments demonstrated that there were decreased levels of pre- and post-synaptic proteins in the hippocampus, diffuse loss of myelin staining and atrophy of the pyramidal layers of hippocampal cornu ammonis 1 (CA1). Estriol treatment prevented decreases in excitatory synaptic transmission and lessened the effect of EAE on PPF. In addition, estriol treatment prevented several neuropathological alterations that occurred in the hippocampus during EAE. Cross-modality correlations revealed that deficits in excitatory synaptic transmission were significantly correlated with reductions in trans-synaptic protein binding partners known to modulate excitatory synaptic transmission. To our knowledge, this is the first report describing a functional correlate to hippocampal neuropathology in any MS model. Furthermore, a treatment was identified that prevented both deficits in synaptic function and hippocampal neuropathology.

More than 50% of MS patients experience cognitive deficits,[1,2] and hippocampal-dependent learning and memory is often impaired.[3,4] Hippocampal atrophy has been shown in MS patients using *in vivo* MRI,[5,6] and this atrophy was correlated with impaired performance on visuospatial memory testing, a hippocampal-dependent function.[7] Data from post-mortem studies in MS have demonstrated that hippocampal demyelination and neuropathology occur.[8,9] This demyelination has been associated with changes in molecules modulating synaptic integrity, axonal transport and glutamate homeostasis.[10] Although evidence from both *in vivo* MRI and post-mortem pathological studies indicates that hippocampal pathology occurs during MS, the relationship between such pathology and dysfunction remains unclear. Moreover, there are currently no treatments available to prevent hippocampal dysfunction during MS.

Demyelination, axonal loss and inflammation have been demonstrated within the central nervous system of mice with experimental autoimmune encephalomyelitis (EAE). Although most EAE studies have focused on spinal cord, only

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recently have studies investigated neurodegeneration within the brain.[11–14] The functional correlates of these described pathologies in brain have not been elucidated. Specifically, little is known about the pathology that occurs in the hippocampus during EAE, and an understanding of how it may lead to dysfunction is lacking.

In MS, estriol, an estrogen elevated during pregnancy, is a therapeutic candidate because it has widespread effects on the immune system and the central nervous system.[15] Studies have shown that MS patients have significantly decreased relapse rates during the third trimester of pregnancy, when estriol levels are most elevated, and that relapse rates rebound during the postpartum period coinciding with an abrupt decline in serum estriol levels.[16] In non-pregnant MS patients, estriol treatment significantly reduced gadolinium-enhancing lesion number and volumes measured by MRI.[17] The study further showed that estriol treatment significantly improved cognitive function as measured by the paced auditory serial addition task (PASAT) in relapsing-remitting MS patients; however, this could have been due, at least in part, to a practice effect.

Estrogen treatment can have both anti-inflammatory[18,19] and neuroprotective effects during EAE.[20,21] Estrogen treatment is clinically beneficial in several neurodegenerative disease models including those for Alzheimer's and Parkinson's diseases,[22] and in other conditions such as traumatic brain injury and stroke.[23,24] In vitro and in vivo studies have used estrogen to protect neurons against degeneration due to excitotoxicity, oxidative stress and apoptosis.[25–27] Finally, estrogen treatment may affect hippocampal learning and memory, as previous studies have shown that estrogen treatment in rodents led to upregulated synaptic protein synthesis,[28] increased activation of neuronal signaling cascades[29] and increased dendritic arborizations.[30] Evidence demonstrates that estrogen treatment enhanced long-term potentiation, altered neuronal excitability and increased synaptic transmission in the hippocampus.[31–33]

In this study, we determine whether hippocampal synaptic protein complexes known to orchestrate functional synaptic transmission and the excitatory synaptic transmission itself are affected during EAE. Furthermore, we determine if estriol, a candidate hormone treatment, is capable of preventing hippocampal synaptic pathology and corresponding functional changes in synaptic transmission.

MATERIALS AND METHODS
Animals and Treatment
Gonadally intact female C57Bl/6 mice, aged 12–16 weeks, were bred in-house from animals purchased from Jackson Laboratories (Bar Harbor, ME, USA). All animals were maintained in accordance with the guidelines of the UCLA Chancellor's Animal Research Committee (ARC), and the PHS Policy on Humane Care and Use of Laboratory Animals. At 1 week before disease induction, 90-day release pellets of estriol (ESTRIOL) at 5 mg dose, as well as placebo (PLAC)

pellets that contain carrier binder alone (Innovative Research of America, Sarasota, FL, USA), were implanted into the scapular region subcutaneously.

EAE Induction and Clinical Disease Scoring
At 1 week after pellet implantation, active EAE was induced by immunizing with 200 μg of myelin oligodendrocyte glycoprotein (MOG) peptide, amino acids 35–55 (Chiron Mimotopes, San Diego, CA, USA) and 300 μg of *Mycobacterium tuberculosis* in complete Freund's adjuvant over two sites: the right draining inguinal and axillary lymph nodes. After 7 days, a booster immunization was delivered subcutaneously over the contralateral draining lymph nodes, as described.[34] For MOG immunizations, mice were temporarily anesthetized with isofluorane. In addition, EAE mice received intraperitoneal injections of *Bordatella pertussis* toxin (500 ng/mouse; Ptx) on days 0 and 2. Mice were monitored daily, and clinical disease severity was measured using the standard EAE grading scale.[35] Clinical scores were averaged across all animals per day, yielding a mean clinical disease index per group.

Slice Electrophysiology
Mice were randomly selected for electrophysiological recording, daily, corresponding to EAE days 21 through 45. Hippocampi were obtained from mice that were deeply anesthetized with halothane and then killed by cervical dislocation. The brain was removed and placed in cold (4 °C), oxygenated (95% O_2/5% CO_2) artificial CSF (aCSF) containing 124 mM NaCl, 25 mM Na_2HCO_3, 4.4 mM KCl, 1 mM NaH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$ and 10 mM glucose. The left hemisphere was collected for subsequent immunohistochemical staining (see below) and the hippocampus from the right hemisphere of each animal was then dissected free from the rest of the brain. The right hippocampus was then cut into 400 μm thick slices perpendicular to its long axis and 3–4 slices from the extreme dorsal and ventral ends were discarded. The remaining slices were maintained in interface-type recording chambers perfused at a constant rate (2–3 ml/min) with a warmed (30 °C), oxygenated aCSF and allowed to recover for at least 2 h before each experiment. Low-resistance (5–10 MΩ) glass microelectrodes filled with aCSF were placed into stratum radiatum of the hippocampal cornu ammonis 1 (CA1) region to record field excitatory postsynaptic potentials (fEPSPs). Presynaptic stimulation pulses were delivered once every 50 s to the Schaffer collateral/commissural fibers via a bipolar nichrome wire stimulating electrode. Basal synaptic transmission was examined by generating input/output curves where fiber volley amplitude (input) and fEPSP slopes (output) were compared across a range of presynaptic fiber stimulation intensities.[36–38] To assess short-term plasticity, hippocampal slices were stimulated to trigger half-maximal (50%) fEPSPs. Paired-pulse facilitation (PPF) was measured by delivering pairs of presynaptic fiber stimulation pulses with varying interpulse intervals (25, 50, 100, 200 and

275 ms) and measured as the ratio of the slope of the fEPSP evoked by the second stimulation pulse relative to that produced by the first stimulation pulse. After a 20-min baseline recording, long-term potentiation (LTP) was elicited with high-frequency stimulation, where two trains of 100 Hz, each with duration of 1 s, were delivered with an intertrain interval of 10 s. LTP was monitored and recorded for 60 min after high-frequency stimulation (HFS). Data acquisition and analysis were performed using the Experimenter's Workbench software package from DataWave Technologies (Loveland, CO, USA).

Immunohistochemistry

Left hemispheres were collected and fixed overnight in 4% paraformaldehyde, for each animal, and then transferred to a 30% sucrose/saline solution. Once tissues from all animals in the experiment had been collected, all samples were gelatin-embedded, frozen and sliced thin using a cryostat at $-20\,°C$, as previously described.[34] To determine hippocampal CA1 volume, tissue sections were uniform randomly collected and nissl stained using standard protocols. Specifically, every fifth sagittal 20 μm-thick tissue section was collected to sample $\sim$640 μm of the left hemisphere (from Bregma lateral co-ordinates 0.12 mm to 1.08 mm; from Paxinos and Franklin Mouse Brain Atlas[39]). For all other immunostaining conditions, three sagittal sections were used from each mouse in each condition. Tissue was first permeabilized in either 2% normal goat or normal rabbit serum (NGS or NRS) in 0.3% Triton X-100 in phosphate buffer solution for 30 min at room temperature. Sections were then blocked in 10–15% NGS/NRS for 2 h at room temperature. To detect microglia, myelin basic protein and synaptic proteins, the following primary antibodies were used: polyclonal anti-Iba1 1:1000 (Wako Chemicals, Richmond, VA, USA); monoclonal myelin basic protein (MBP; 1:500); polyclonal anti-Synapsin-1 (Syn-1; 1:500); monoclonal postsynaptic density protein 95 (PSD-95; 1:500; Chemicon, Temecula, CA, USA); polyclonal neurexin IIβ (NRXIIβ; 1:500); and polyclonal neuroligin 1 (NLG1; 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Fluorescent-conjugated secondary antibodies (goat anti-mouse Cy5 (1:750; Chemicon); goat anti-rabbit Cy3 IgG (1:1000; Vector Laboratories, Burlingame, CA, USA); and rabbit anti-goat Cy5 (1:750; Vector Labs) in 2% NGS/NRS in TBS solution) were used to visualize staining. Positive and negative controls were performed for each staining to ensure antibody specificity. 4',6-diamidine-2'-pheynylindole dihydrochloride (DAPI; 1:1000; Invitrogen, Eugene, OR, USA) staining was used in all fluorescence staining conditions to identify nuclear DNA in all cell types.

Microscopy and Stereological Analysis

CA1 volume was estimated using the rigorous Cavalieri method.[40,41] Iba1+ cells morphologically representative of reactive microglia in CA1 were counted using unbiased stereology methods and presented as number of cells by area.[42]

By carefully delineating CA1 regions by anatomical landmarks, the area of myelin and PSD-95 staining within the CA1 were measured as percent area of immunoreactivity, using ImageJ (NIH; http://rsb.info/nih/gov/ij). To quantify Syn-1, NRXIIβ and NLG1 immunoreactive puncta in CA1, puncta size threshold range was determined by measuring puncta size range in several tissues from each staining condition in many animals, and then averaging both upper and lower threshold limits. For Syn-1, this yielded a puncta size range from 0.816 to 3.264 μm^2. NRXIIβ puncta size ranged from 0.1015 to 0.5075 μm^2, and NLG1 puncta size ranged from 0.0508 to 0.5075 μm^2. These ranges were then used to filter for puncta particle size throughout the experiment for all three of these synaptic stains. The number of puncta in each optical image was averaged across z-stack, brain section and animal to attain a composite average of synaptic puncta within the sampled hippocampal area in each condition.

Statistical Analysis

All quantitative measures are presented as mean ± s.e.m. and analyzed using Student's t-test or one-way ANOVA. Post-hoc analyses were conducted only if ANOVA yielded $P < 0.05$, with Bonferroni analysis. Pearson's linear regression analysis was used to determine correlations between electrophysiology and pathology using GraphPad Software Version 4.0 (San Diego, CA, USA). Cross-modality correlations have been made in other previously published work.[43–45]

RESULTS

Active EAE was induced using MOG 35–55 peptide and standard EAE clinical scores were attained, primarily reflecting the level of motor disability. There were three groups: placebo-treated healthy control females; placebo-treated females with EAE; and estriol-treated females with EAE (Figure 1a). Estriol-treated females with EAE had significantly decreased clinical disability scores compared with placebo-treated females with EAE, whereas healthy control females did not display clinical disease (Figure 1b). These findings were consistent with previous work from our lab and others,[18,19,46] demonstrating that estriol treatment ameliorates motor disability in EAE.

Synaptic Transmission and PPF Are Altered in the Hippocampus During EAE
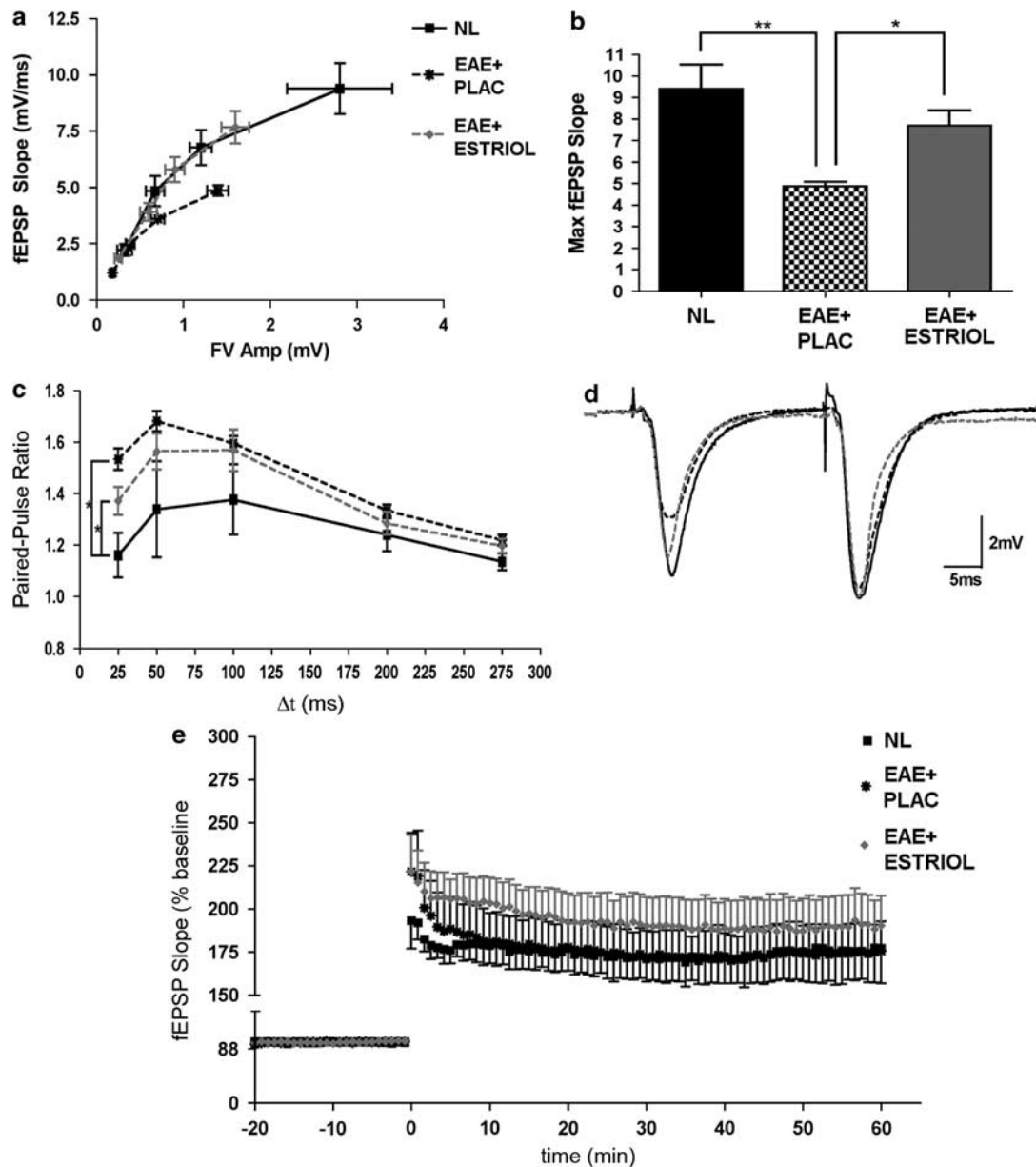
Excitatory synaptic transmission in the hippocampus during EAE has not been previously investigated. Synaptic transmission is dependent on functional and appropriately located synaptic protein families. Thus, basal synaptic transmission and PPF were assessed using in vitro hippocampal slices. To determine if EAE caused an effect in excitatory synaptic transmission, input/output (IO) relationships were compared in hippocampal slices from the right hemispheres of mice in each experimental group. The amplitude of presynaptic fiber volleys and the slope of the EPSPs evoked by different intensities of Schaffer Collateral fiber stimulation
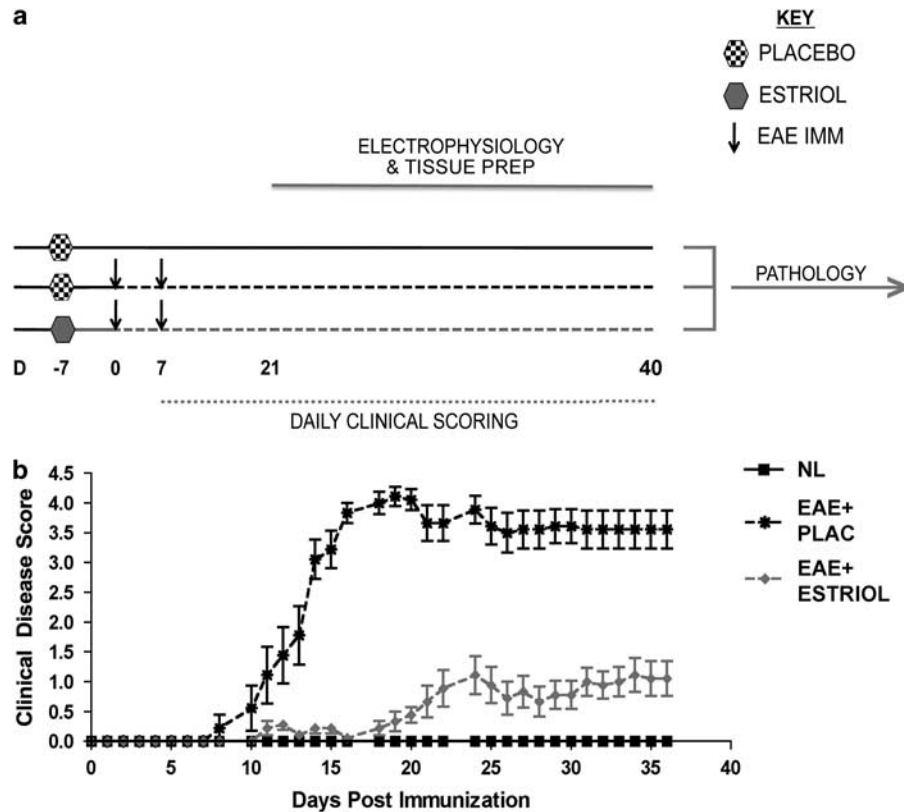
Figure 1 Experimental design and standard clinical scores in EAE. (**a**) Experimental design depicting the timing of placebo or estriol pellet implantation (D −7), EAE induction (D0 and D7), daily clinical scoring (D 7–40) and electrophysiology (D21–40). Pertussis injections (intraperitoneal (i.p.)) were given to both EAE groups on D0 and D2, as part of standard EAE induction (not shown). All mice were age-matched C57Bl/6 adult females, and were uniform randomly selected (one per day) for electrophysiological recording. On each day of electrophysiology, the right hemisphere was prepared for electrophysiology, whereas the left hemisphere of each mouse was prepared for pathology studies and kept until all tissue from all conditions had been collected. (**b**) EAE clinical scores were recorded in placebo-treated (EAE + PLAC, black star, dashed black line) or estriol-treated (EAE + ESTRIOL, gray triangle, dashed gray line) mice as well as in non-EAE induced healthy controls (NL, black square, solid black line). Placebo-treated mice with EAE exhibited a severe clinical disease course, whereas estriol-treated mice exhibited significantly reduced clinical severity. Data are representative of two separate experiments, and experiment has been repeated more four times. Repeated measures ANOVA with *post-hoc* pair-wise comparisons revealed that placebo-treated EAE mice were significantly different from other two groups beginning at D12 of disease, *$P < 0.05$, $n = 5$ per group per experiment.

were analyzed. Here, hippocampal slices from placebo-treated EAE mice had a significantly altered IO curve compared with those from healthy controls (Figure 2a). Hippocampal slices from EAE mice had FV Amps and fEPSP slopes that were both decreased by nearly 50%, suggesting that basal synaptic transmission was significantly impaired during EAE. When treated with estriol, however, hippocampal slices from mice with EAE displayed IO relationships more similar to healthy controls (Figure 2a and b). When maximal fEPSP slopes were compared across the three groups, there was a significant deficit in postsynaptic responses in slices from EAE mice compared with healthy controls, and this decrease in the postsynaptic response was prevented with estriol treatment (Figure 2b). Next, to examine short-term plasticity, we measured PPF at Schaffer collateral fiber inputs onto CA1 pyramidal cells. PPF is inversely correlated with the probability of neurotransmitter release from presynaptic terminals, such that an increase in PPF is indicative of a decrease in transmitter release probability, whereas a decrease

in PPF is indicative of an increase in transmitter release probability. Compared with healthy controls, placebo-treated EAE mice displayed significantly increased PPF at the shortest paired-pulse interval tested (Figure 2c and d). Interestingly, although PPF in slices from estriol-treated EAE mice was also significantly elevated compared with healthy controls, the increase tended to be lower than placebo-treated EAE mice at the same time interval (Figure 2c and d). As hippocampal slices from placebo-treated EAE mice had significantly increased paired-pulse ratios, our results suggest that EAE caused a decrease in the probability of presynaptic neurotransmitter release. With estriol treatment during EAE there was a trend of partially restored release probability. To examine if EAE impaired synaptic plasticity, LTP was induced with HFS (2×100 Hz) in hippocampal slices from mice in each condition. Two separate components of LTP were analyzed: post-tetanic potentiation (PTP; the immediate potentiated response of each slice after HFS stimulation) and early-phase LTP (eLTP; the average of the past 5 min of LTP

Figure 2 Excitatory synaptic transmission and paired-pulse facilitation are altered during EAE. (**a**) Input/output (IO) curves were created by comparing fiber volley amplitude (FV Amp; mV) to field excitatory postsynaptic potential (fEPSP; mV/ms) during four different stimulation intensities of presynaptic fiber stimulation that caused fEPSP responses of 25, 50, 75 and 100% maximal fEPSP amplitude. One-way ANOVA analyses demonstrated that placebo-treated EAE (EAE + PLAC; black star, dashed black line) fEPSP responses were significantly reduced at each point along the IO curve (percentage of maximal fEPSP slope: 25, 50 75 and 100%) in comparison with healthy controls (NL; black squares, solid black line), $P < 0.05$. With estriol treatment during EAE (EAE + ESTRIOL; gray triangle, dashed gray line), the IO curve followed a similar trajectory to healthy controls. (**b**) A histogram demonstrating decreased maximal fEPSP responses in placebo-treated EAE compared with healthy controls, and maximal fEPSPs in estriol-treated EAE mice that were not significantly different from controls. One-way ANOVA revealed a significant effect of disease on maximal fEPSP slope ($P = 0.0029$) and follow-up Bonferroni tests indicated that placebo-treated EAE mice (EAE + PLAC; $n = 15$ slices from 5 mice) were significantly different from healthy controls (NL, $n = 7$ slices from 3 mice; **$P < 0.01$) and also significantly different from estriol-treated EAE (EAE + ESTRIOL, $n = 16$ slices from 5 mice; *$P < 0.05$). (**c**) Paired-pulse facilitation was increased in the hippocampal CA1 region of placebo-treated EAE mice. Paired presynaptic fiber stimulation pulses were delivered with varying interpulse intervals (25, 50, 100, 200 and 275 ms) to elicit postsynaptic responses with amplitudes that were then compared. At an interpulse interval of 25 ms, one-way ANOVA demonstrated that the condition had a significant effect on paired-pulse facilitation ($P = 0.0178$). Bonferroni *post-hoc* analysis showed that placebo-treated mice (EAE + PLAC; black star, dashed black line, $n = 12$ slices from 5 mice) had significantly greater paired-pulse facilitation (*$P < 0.05$) compared with healthy controls (NL; black square, solid black line, $n = 6$ slices from 3 mice). Estriol-treated mice (EAE + ESTRIOL; gray triangle, dashed gray line, $n = 11$ slices from 5 mice) also had significantly increased PPF compared with healthy controls. (**d**) Sample waveforms depict representative paired-pulse responses recorded from animals in each experimental condition at a 25-ms interpulse interval (NL: black solid line; EAE + PLAC: dashed black line; and EAE + ESTRIOL: gray dashed line). (**e**) Neither the post-tetanic potentiation (PTP; the first response after high-frequency stimulation) nor the early phase of LTP were significantly affected by EAE, as analyzed by one-way ANOVAs. Furthermore, one-way ANOVAs revealed that estriol treatment during EAE did not have an effect on PTP or eLTP.
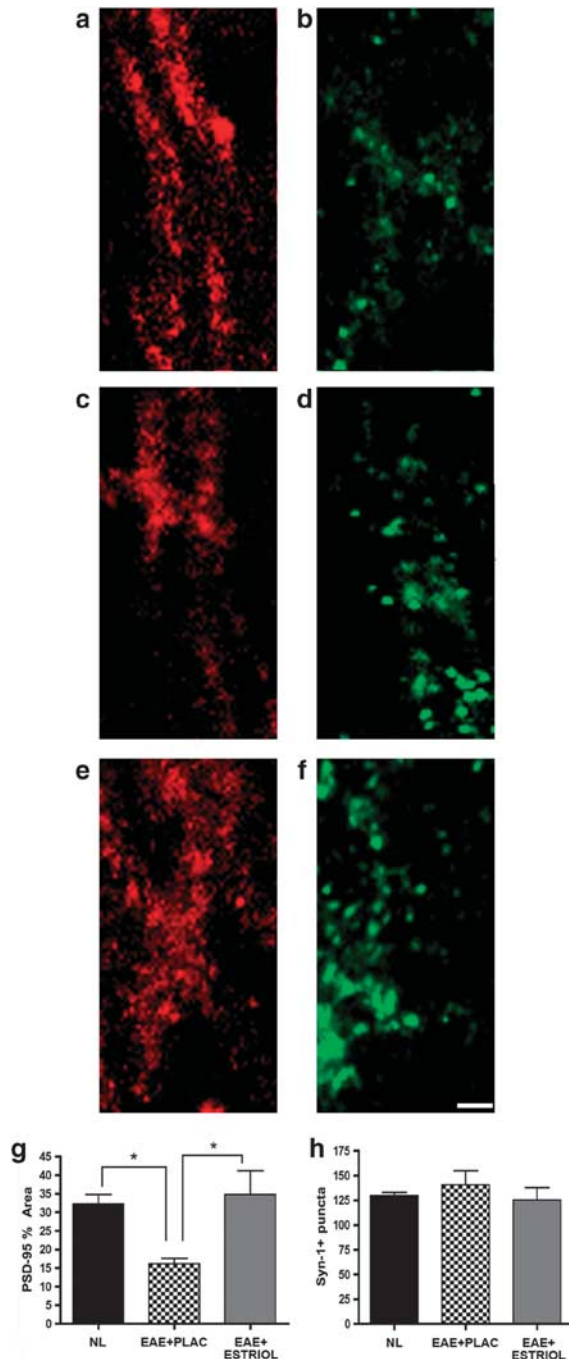
Figure 3 Hippocampal PSD-95 levels are decreased in EAE and preserved with estriol treatment. (a–f) Fluorescent images depict the *stratum radiatum* with the CA1 region of representative hippocampal slices from healthy (**a**, **b**), placebo-treated EAE (**c**, **d**) and estriol-treated EAE (**e**, **f**) mice, where PSD-95 (**a**, **c**, **e**; Cy5-red), and Synapsin-1 (**b**, **d**, **f**; Syn-1; Cy3-green) staining are shown at × 60 magnification. During EAE, PSD-95 percent area was significantly decreased in the CA1 region of the hippocampus (**c**) compared with healthy controls (**a**). This decrease was prevented with estriol treatment during EAE (**e**), which is quantified in graph **g**. One-way ANOVA and Bonferroni *post-hoc* analysis revealed statistical significance in PSD-95 percent area where $*P = 0.0324$, $n = 5$ per group. Average number of presynaptic Syn-1+ puncta within the CA1 was not significantly different across conditions (**h**). These experiments were repeated twice with similar results. Scale bar, 1 μm.

in each slice). When compared across groups, PTP was not significantly affected by disease or by estriol treatment during disease. eLTP was also unaffected by EAE or estriol treatment during EAE (Figure 2e).

Pre- and Post-synaptic Proteins Are Differentially Affected in the Hippocampus During EAE and Estriol Treatment of EAE

Synaptic health and transmission are critical to hippocampal functions including learning and memory. Because excitatory synaptic transmission and PPF were both affected during EAE, we next assessed whether synaptic proteins were also affected in the hippocampus during EAE. Presynaptic (Syn-1 and NRXIIβ) and postsynaptic (PSD-95 and NLG1) protein immunoreactivity was measured in the hippocampus of normal, placebo-treated EAE and estriol-treated EAE mice. The percent area of PSD-95 staining was significantly decreased in EAE mice compared with healthy controls (Figure 3a *vs* c, and graph g). Estriol treatment during EAE reversed this effect on PSD-95 (Figure 3e and graph g). In contrast, the number of Syn-1 puncta was not significantly reduced during EAE (Figure 3b, d, f and graph h).

Recent evidence has demonstrated that PSD-95 can bind to NLG1 to modulate presynaptic release probability in a retrograde trans-synaptic fashion by binding to β-neurexin II.[47] As we found that PSD-95 levels were significantly decreased in the hippocampus during EAE, we next investigated whether EAE affected levels of presynaptic NRXIIβ and postsynaptic NLG1. We found that immunoreactive NRXIIβ puncta were significantly decreased in hippocampi from placebo-treated EAE compared with healthy controls (Figure 4a and b). Estriol treatment of EAE mice prevented this decrease in NRXIIβ and resulted in a similar amount of immunoreactive puncta within hippocampus as that found in healthy control mice. Immunoreactive NLG1 puncta were also significantly decreased in placebo-treated EAE compared with healthy controls, and estriol treatment of EAE also prevented this decrease in NLG1 (Figure 4c and d).

Demyelination and Microglial Activation Occur in the Hippocampus During EAE

Demyelination is a classic neuropathological finding in spinal cords of mice with EAE and often leads to deficits in sensory and motor function. Demyelination has been observed in the hippocampus of MS patients[10] generally as focal demyelinating lesions. We have demonstrated in a previous report that there is a diffuse reduction in myelin staining in the hippocampus during EAE.[34] Thus, myelin staining was assessed in this study. MBP fluorescence intensity measurements demonstrated a significant reduction in myelin immunoreactivity within the delineated CA1 region of placebo-treated EAE mice, compared with healthy controls (Figure 5a, b and d). Estriol treatment during EAE significantly preserved myelin staining in the CA1 region
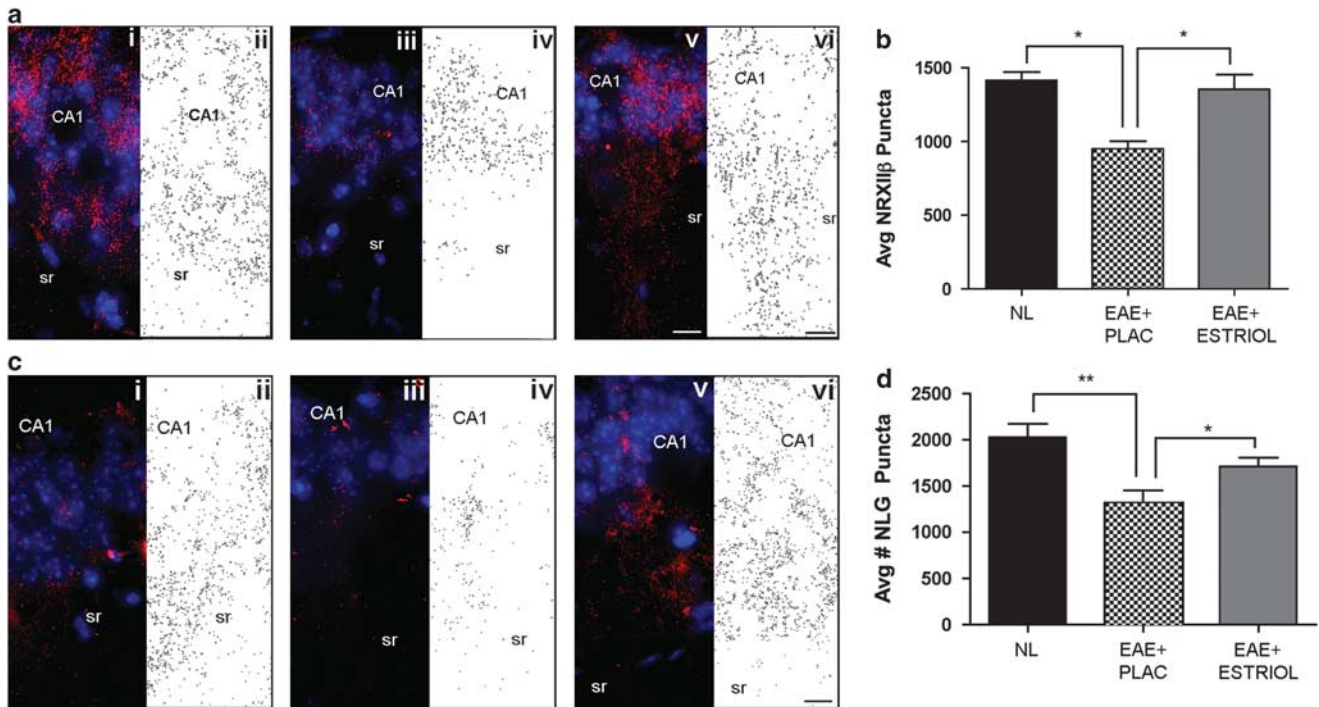
Figure 4 Presynaptic β-neurexin II (NRXIIβ) and postsynaptic neuroligin-1 (NLG1) are decreased during EAE, but preserved with estriol treatment. Synaptic puncta were measured in the hippocampal CA1 region by counting immunoreactive NRXIIβ puncta (**a**, **b**) and NLG1 puncta (**c**, **d**) in healthy control (panels i, ii), placebo-treated EAE (panels iii, iv) and estriol-treated EAE mice (panels v, vi), respectively. Pseudo-colored confocal images depict synaptic staining (either NRXIIβ or NLG1, Cy3-red) in hippocampus at $\times 60$ magnification, whereas black and white images show outlined puncta from respective confocal images. (**b**) One-way ANOVA revealed that groups differed significantly in quantity of NRXIIβ puncta ($P = 0.005$), and Bonferroni *post-hoc* test indicated that placebo-treated EAE mice (EAE + PLAC, $n = 5$ mice) had significantly decreased (*$P < 0.01$) numbers of NRXIIβ puncta compared with healthy control (NL, $n = 5$ mice) and estriol-treated EAE mice (EAE + ESTRIOL, $n = 4$ mice). (**d**) Simultaneously, NLG1 puncta quantities were also significantly different across conditions (one-way ANOVA, $P = 0.0056$) with follow-up tests indicating significant difference between healthy controls (NL, $n = 5$ mice) and placebo-treated EAE mice (EAE + PLAC, **$P < 0.01$, $n = 5$ mice), as well as significant difference between placebo-treated EAE and estriol-treated EAE mice (EAE + ESTRIOL, *$P < 0.05$, $n = 5$ mice). These experiments were repeated twice and similar results were found. Scale bars, 10 μm.

(Figure 5c and d) compared with placebo-treated EAE mice, but myelin staining levels were not similar to normal controls.

Reactive microglia with infiltration of T lymphocytes and macrophages characterize the inflammation that occurs in spinal cords of EAE mice. Within the hippocampus, however, we have previously demonstrated that immune cell infiltrates are minimal, with inflammation limited to resident reactive microglia.[34] Reactive microglia and the inflammatory molecules they produce have also been shown to detrimentally affect synaptic transmission.[14] Iba1 is a protein highly expressed by resident microglia in the hippocampus.[48] Iba1 is also expressed by macrophages, and the two cell types may be distinguished by morphology, as we have previously described,[49] Thus, Iba1 staining was used to label, count and morphologically characterize cells of microglial/macrophage lineage. Hippocampal tissue sections from placebo-treated EAE mice demonstrated a trend for elevated numbers of highly branched, ramified microglia compared with healthy controls, which had small, finely branched quiescent microglia (Figure 5a, b and graph e). Estriol treatment in EAE mice tended to prevent this

increase in ramified microglia (Figure 5c and graph e). Within the hippocampus, we did not find large, globoid Iba1-positive cells, suggesting that inflammation in the hippocampus consists primarily of reactive microglia and not immune cell infiltrates.

CA1 Pyramidal Layer Volume Is Decreased During Disease and Preserved with Estriol Treatment

The hippocampal CA1 region undergoes atrophy in humans with MS.[7] As such, we next determined if atrophy was occurring in the hippocampal CA1 during, where altered synaptic transmission had also been found. CA1 volume was rigorously assessed using the Cavalieri method of volume estimation for histopathological data. EAE caused a significant decrease in CA1 pyramidal layer (CA1 *pyr*) volume compared with healthy controls (Figure 6a, b and d). Importantly, estriol treatment during EAE prevented CA1 pyramidal layer volume loss and preserved CA1 layer volume to levels similar to healthy controls (Figure 6c and d).
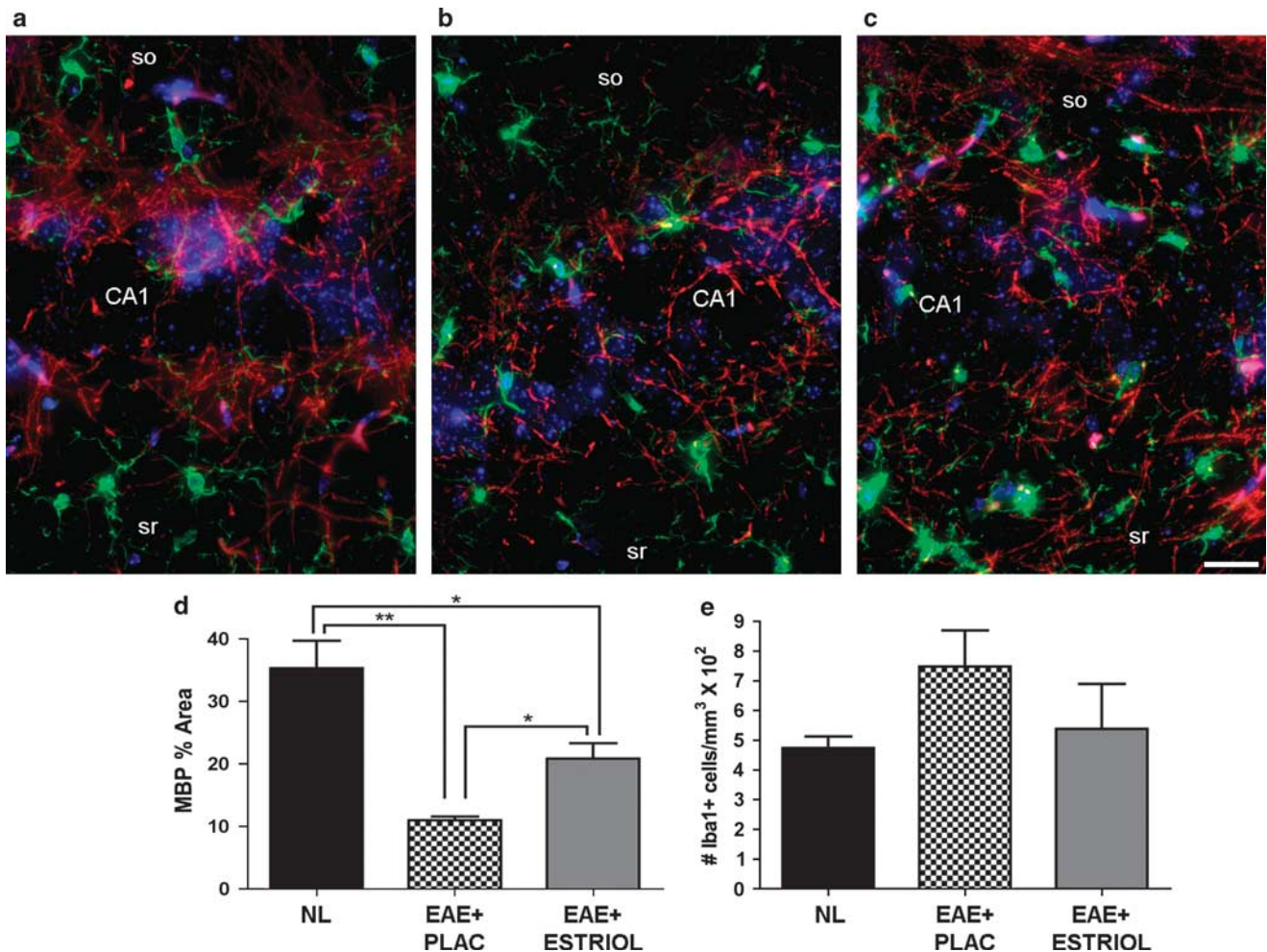
Figure 5 Decreased myelin staining and increased microglial activation occur within the hippocampus during EAE, and estriol treatment preserves myelin staining. (a–c) Pseudo-colored confocal fluorescent images depict myelin basic protein immunoreactivity (MBP, Cy5-red) and cells of microglial/macrophage lineage stained with Iba1 (Cy3-green) and DAPI (blue) in the CA1 region within healthy (a), placebo-treated EAE (b) and estriol-treated EAE (c) mice at ×40 magnification. (d) One-way ANOVA revealed significant differences in myelin staining across conditions, $P = 0.003$, $n = 5$ mice per group, and Bonferroni *post-hoc* tests indicated that EAE caused a significant reduction in myelin staining within the CA1 region of the hippocampus in placebo-treated mice (EAE + PLAC, **$P < 0.01$, $n = 5$ mice) compared with myelin staining in hippocampus of healthy control mice (NL, $n = 5$ mice). Estriol-treated EAE mice (EAE + ESTRIOL, $n = 5$ mice) had myelin levels significantly increased compared with placebo-treated EAE mice (*$P < 0.05$), but were still significantly different from myelin staining levels in healthy controls. Analysis was conducted on ×10 magnification images where MBP percent area represents total MBP immunoreactivity as a percent of total delineated CA1 area imaged. Iba1 + cells with morphology characteristic of reactive microglia were relatively low in healthy controls (a), and increased in placebo-treated EAE mice (b). However, the number of Iba1 + cells was not significantly different in estriol-treated EAE mice (c). The number of Iba1 + cells per mm^3 were counted and presented as whole numbers (e). Data are representative of two experiments. Scale bar, 10 μm.

Neuropathological Outcome Measures Are Significantly Correlated with Changes in Electrophysiological Outcome Measures

Because we found that excitatory synaptic transmission was altered in hippocampal slices from placebo-treated EAE mice and also found neuropathology in tissue from those same animals, we next used cross-modality correlations to determine if functional and pathological outcomes were related during disease state. Specifically, we investigated if changes in PSD-95 and NRXII staining levels correlated with changes in excitatory synaptic transmission. Interestingly, PSD-95 percent area in disease states was significantly correlated to

fEPSP responses during disease states (Figure 7a). Furthermore, the number of immunoreactive NRXII puncta was also significantly correlated to fEPSP (Figure 7b) during disease states. Thus, these cross-modality correlations together demonstrate that excitatory synaptic transmission during EAE coincides with levels of PSD-95 and NRXIIβ in the hippocampus during EAE.

DISCUSSION

The results of the present study show, for the first time, that excitatory synaptic transmission and PPF are significantly impaired in the hippocampus during EAE and that estriol
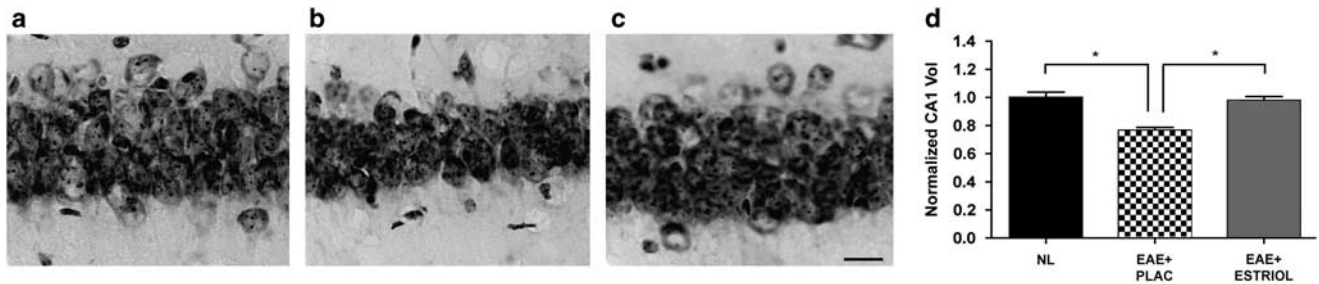
Figure 6 Hippocampal CA1 atrophy occurs during EAE and estriol treatment prevents this. (a–c) Nissl-stained hippocampal sections representative of normal (NL; **a**), placebo-treated EAE (EAE + PLAC; **b**) and estriol-treated EAE (EAE + ESTRIOL; **c**) mice depict the CA1 pyramidal layer at × 40 magnification. Compared with healthy controls, placebo-treated EAE mice had significantly reduced CA1 pyramidal layer volume, quantified in panel **d**. With estriol treatment during EAE, the CA1 pyramidal volume was preserved to levels similar to those in controls. One-way ANOVA and Bonferroni *post-hoc* analysis revealed significant difference between three groups (*$P = 0.05$, $n = 5$ mice per group), scale bar, 10 μm. This finding has been confirmed by repeated experiments.
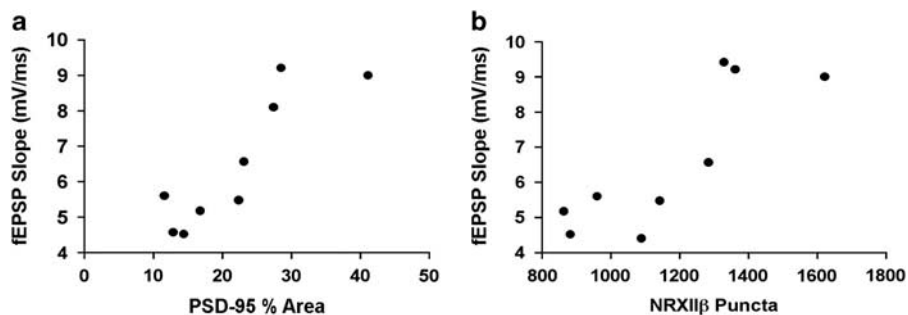
Figure 7 Decreased pre- and post-synaptic protein levels are significantly correlated with electrophysiological changes during EAE. Cross-modality correlations were conducted to determine potential neuropathological substrates underlying changes in excitatory synaptic transmission and paired-pulse facilitation during EAE. (**a**) PSD-95 percent area was significantly correlated to maximal fEPSP slope throughout the experiment, where the correlation coefficient rho $= 0.8767$, $P = 0.019$, $n = 9$ mice total. (**b**) The average number of presynaptic NRXIIβ puncta was also significantly correlated to postsynaptic responses (fEPSP) where rho $= 0.8396$, $P = 0.0046$, $n = 9$ mice total.

treatment is capable of preventing this. Furthermore, we demonstrate that these functional deficits occur within tissue that has neuropathological changes in expression of synaptic proteins known to play an important role in excitatory transmission in the hippocampus (PSD-95, NRXIIβ and NLG1). Interestingly, estriol treatment prevented these changes in synaptic protein expression.

Our data show a reduction of both pre- and post-synaptic electrophysiological responses in hippocampal slices of mice with EAE. Presynaptic facilitation is inversely related to synaptic vesicle release probability at presynaptic terminals.[50,51] As we found elevated PPF during disease, this suggested that EAE decreased the probability of presynaptic vesicle release. Presynaptic terminal structure and protein composition can directly mediate changes in the probability of synaptic vesicle release.[52,53] Thus, it is plausible that EAE leads to detrimental changes in presynaptic terminal structure and protein composition. Postsynaptic excitatory responses were also reduced in this study, and thus EAE may cause simultaneous alterations to pre- and post-synaptic terminals that lead to impaired synaptic transmission.

EAE induced a reduction in protein levels of trans-synaptic binding partners. PSD-95, NRXIIβ and NLGI are distinct, interacting scaffolding proteins known to have important roles in excitatory synaptic transmission.[54] PSD-95 levels were significantly decreased in the hippocampus of mice with EAE. PSD-95 is abundantly expressed in healthy excitatory synapses and has been shown to recruit diverse proteins to sites of synaptic adhesion[55] and promote trans-synaptic signaling.[56] PSD-95 also has extensive interactions with NLG1 (a postsynaptic cell adhesion protein) at excitatory synapses[57] and with NRXIIβ (a presynaptic cell adhesion protein) to mediate presynaptic terminal assembly.[58] Indeed, neurexin/neuroligin trans-synaptic binding is necessary for proper synapse formation, stability, maturation and function.[59] Both NRXIIβ and NLG1 protein levels were reduced in the hippocampus during EAE and, moreover, cross-modality correlations demonstrated that the reductions in these protein levels were correlated with reductions in basal synaptic transmission and PPF. PSD-95 and neuroligin can directly modulate the release probability of neurotransmitter vesicles in a retrograde fashion via direct binding with neurexin.[47] Thus, the changes in these synaptic protein levels during EAE may affect probability of vesicle release, and result in impairment of hippocampal function.

Estrogens are known to have extensive effects on the hippocampus in healthy and disease states, making them likely candidates for neuroprotective treatments. Estrogens promote synaptogenesis[60] and can enhance synaptic transmission in healthy states[61] by facilitating presynaptic and postsynaptic plasticity.[62,63] In addition, estrogens can significantly increase expression of synaptic proteins required for functional synaptic transmission, including PSD-95[64] and neuroligin.[65] Besides providing beneficial support to synaptic integrity and function in healthy conditions, estrogens have also been shown to induce other neuroprotective mechanisms in other diseases.[66] In animal models of Alzheimer's disease, estrogen treatment reduced β-amyloid protein accumulation,[67] promoted neuron viability[68] and enhanced NMDA-mediated synaptic transmission and long-term plasticity.[63] In EAE, estrogen treatment has been shown to reduce clinical disease severity[18,19] through both neuroprotective and anti-inflammatory actions.[20,21,42] As EAE principally affects ambulation, the majority of these studies have been limited to spinal cord pathology. One recent publication has examined estrogen-mediated neuroprotection in the corpus callosum[12] and found that estrogen treatment prevented callosal axon loss and preserved axonal conductance across the corpus callosum. Our study is the first to report that estriol, a pregnancy estrogen, is capable of preventing EAE-mediated impairment hippocampal function and neuropathology. Important evidence from human clinical trials in MS suggests that estriol treatment may be beneficial in clinical application[15,17,69] and further investigation in clinical trials is underway (http://clinicaltrials.gov/ct2/show/NCT00451204). This report suggests that estriol treatment should be further investigated for its potential role in preventing hippocampal dysfunction and neuropathology in MS.

It remains to be determined where and how estriol might act to prevent the pathological changes that occur in the hippocampus during EAE. To understand the cellular and molecular mechanism of estrogen-mediated protection in the hippocampus during EAE, treatment with estrogen-receptor-specific ligands must be investigated. Estrogen-mediated protection, like other estrogenic actions, is mediated primarily through estrogen receptors, ERα and ERβ, although other non-genomic mechanisms have been identified. ERα and ERβ have distinct tissue distributions,[70] which may cause some tissue selectivity using selective estrogen receptor modifiers (SERMs). The two receptors act synergistically in some tissues, whereas they act antagonistically in others. These tissue-specific differences are thought to be because of tissue-specific differences in transcription factor activation upon ER-ligand binding. In a previous publication, our lab has shown that ERα ligand (PPT) treatment ameliorated clinical disease score throughout disease, whereas treatment with an ERβ ligand (DPN) had no effect in early EAE, but promoted recovery during the late phase of disease.[21] In addition, EAE mice treated with ERα ligand had significantly reduced CNS inflammation, whereas EAE mice treated with ERβ ligand did

not. Interestingly, treatment with either the ERα or the ERβ ligand was neuroprotective, as evidenced by axonal sparing within the white matter of the spinal cord. By using ER-specific ligands, the anti-inflammatory effects were dissociated from the neuroprotective effects of estrogen treatment during EAE, as only ERα ligand treatment significantly reduced inflammation in the spinal cord. These data suggested that the neuroprotective effects of estrogen treatment are not necessarily dependent on anti-inflammatory properties, but rather may be mediated by direct effects of estrogen binding to estrogen receptors on cells within the central nervous system. Estrogen receptors have been detected on several CNS cells, including neurons,[71,72] astrocytes,[73] oligodendrocytes and microglia.[74] Recently, it was shown that when ERα was selectively removed from astrocytes, but not neurons, the protection from spinal cord pathology in ERα ligand-treated EAE mice was lost. Both ERα and ERβ are expressed in the hippocampus.[75] Future studies using CNS cell-specific ER knockouts are needed to determine whether the astrocyte or other CNS cell is the target of estriol-mediated protection in the hippocampus during EAE.

In conclusion, this is the first report demonstrating that EAE leads to impaired synaptic transmission and corresponding changes in synaptic protein levels within the hippocampus. Moreover, our data argue strongly that estriol treatment is neuroprotective in the hippocampus by preventing both dysfunction and neuropathology. Although there are always limitations to the conclusions that one can draw from animal models of disease, this report provides a foundation for further studies that focus on hippocampal synaptic proteins as they relate to hippocampal-dependent cognitive impairment in MS.

DISCLOSURE/CONFLICT OF INTEREST
The authors declare no conflict of interest.

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