Supporting Information

Spence et al. 10.1073/pnas.1103833108

SI Materials and Methods

Animals. All mice were on a C57BL/6 background achieved by at least 10 generations of back crossing. Astrocyte-ERα-CKO (conditional gene knockout) were generated by crossing mice of mGFAP-Cre line 73.12 (1), with mice carrying an estrogen receptor-α (ERα) gene in which exon 3 was flanked by loxP sites ($ERα^{flox/flox}$) were the generous gift of Pierre Chambon (Faculty of Medicine, University of Louis Pasteur, Strasbourg, France) (2). Neuron-ERα-CKO were generated by crossing rat neuronal specific enolase (rNSE)-Cre mice (3, 4), with mice carrying an ERα gene in which exon 3 was flanked by loxP sites ($ERα^{flox/flox}$) (2). Animals were maintained under standard conditions in a 12-h dark/light cycle with access to food and water ad libitum. All procedures were done in accordance to the guidelines of the National Institutes of Health and the Chancellor's Animal Research Committee of the University of California, Los Angeles Office for the Protection of Research Subjects.

Astrocyte Culture. Purified primary astrocyte cultures were prepared from individual postnatal day 1 to 3 male and female mouse pups, as described previously (5). Cortices were dissected and dissociated aseptically and cells plated in 35-mm six-well plates (1.2–1.6 hemispheres/12 mL per plate; Falcon Primaria, BD Biosciences). Plating media consisted of L-glutamine–free DMEM (Invitrogen) supplemented with 10% FBS (HyClone), 10% iron-supplemented calf serum (HyClone), 2 mM L-glutamine (Invitrogen), 50 IU/mL penicillin and 50 μg/mL streptomycin (Invitrogen), and 10 ng/mL epidermal growth factor (Invitrogen). Once astrocytes reached confluence (after ~7 d), cultures were treated with 8 μM cytosine β-D-arabinofuranoside (5–6 d) to antagonize microglial growth. Thereafter, cultures were maintained in L-glutamine–free DMEM containing 10% calf serum, 50 IU/mL penicillin and 50 μg/mL streptomycin, and 2 mM L-glutamine. One day before experimentation, cultures were treated with 75 mM L-leucine methyl ester (60–90 min) to further reduce microglia contamination (5). All cultures were grown and maintained at 37 °C in a humidified incubator containing 6% CO_2.

RT-PCR. Total RNA was isolated and purified using RNeasy Mini Kit (Qiagen) and was resuspended in RNase-free H_2O (Ambion). RNA was quantified spectrophotometrically at 260 nm (Nanodrop) and 0.5 mg reverse-transcribed using reverse transcriptase and oligo $(dT)_{(12\text{-}18)}$, as previously described (5). cDNAs for ERα and β-actin were amplified in a thermal cycler (MJ Research) using amplimers for mouse ERα (5′-TTACGAAGTGGGCATGATGA-3′ and 5′-ATAGATCATGGGCGGTTCAG-3′) and β-actin (5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′). ERα was amplified for 33 cycles (94 °C/30 s, 58 °C/45 s, 72 °C/60 s) and β-actin was amplified for 23 cycles (94 °C/30 s, 65 °C/45 s, 72 °C/60 s). PCR products (~200 and 540 bp, respectively) were separated in a 2% agarose gel containing ethidium bromide (0.5 mg/mL) and visualized with a UV transilluminator (Cole Palmer). Images were processed using Adobe Photoshop.

Western Blot. Ovary tissue and primary hypothalamic astrocyte cultures were prepared from two female and two male 40-d-old WT and CKO mice, as previously described (6). Briefly, a hypothalamic block was isolated with the following boundaries: the rostral extent of the optic chiasm, rostral extent of the mammillary bodies, lateral edges of the tuber cinereum, and the top of the third ventricle. Hypothalamic tissue was dissociated with 2.5% of trypsin (Invitrogen) and a fire-polished glass Pasteur pipette. Cultures were maintained at 37 °C with 5% CO_2 and grown in DMEM/F12 media (Mediatech) with 10% FBS (HyClone) and 1% penicillin-streptomycin (Mediatech) for 14 to 20 d. Once grown to confluency, astrocyte cultures were used for the experiment.

Ovary tissue and astrocyte cells were homogenized in RIPA Lysis Buffer (Santa Cruz Biotechnology) containing the following proteases inhibitors: 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/mL pepstatin, 1 μg/mL leupeptin, 1 μg/mL aprotinin, and 1 mM sodium orthovanadate (all inhibitors were from Santa Cruz Biotechnology). After incubating for 30 min on ice, the samples were centrifuged (10,000 × g for 2 min at 4 °C). Protein concentrations of the supernatant were determined with the Bradford Assay (Bio-Rad). Samples containing equal amounts of protein were combined with sample buffer, boiled, separated by 10% SDS polyacrylamide gel, and then transferred to PVDF membranes (Amersham Biosciences). For the detection primary antiestrogen receptor-α (1:1,000; Upstate Biotechnology, Inc.) and secondary donkey anti-rabbit IgG (H+L) antibodies (1:5,000; Jackson ImmunoResearch) were used. Bands were visualized using an enhanced chemiluminescence (ECL) kit and ECL Hyperfilm (GE Healthcare). For determination of the molecular weight of the proteins, samples were run with Biotinylated Protein Ladder (Cell Signaling). Images were processed using Ado be Photoshop.

Adoptive Experimental Autoimmune Encephalomyelitis and Hormone Manipulations. C57BL/6 donor animals were immunized subcutaneously with myelin oligodendrocyte glycoprotein, amino acids 35 to 55 (200 μg per animal, American Peptides) emulsified in complete Freund's adjuvant (CFA), supplemented with *Mycobacterium tuberculosis H37Ra* (200 μg per animal; Difco Laboratories), over four sites, and drained by inguinal and auxiliary lymph nodes in a total volume of 0.1 mL per mouse. Immunized mice had lymph node cells cultured in 24-well plates at a concentration of 3×10^6 cells/mL of complete RPMI medium. Cells were stimulated with 25 μg/mL MOG, peptide 35–55, and 20 ng/mL recombinant mouse IL-12 (BD Biosciences and Biolegend) for 72 h. On the third day of culture, lymph node cells were washed with 1× PBS and each recipient mouse received 3×10^7 cells in 0.3 mL ice-cold PBS by intraperitoneal injection. Recipient female C57BL/6 WT and CKO mice had been gonadectomized at 4 wk of age, and had EAE induced by adoptive transfer at 8 wk of age. Recipient mice were either treated every other day with the ERα ligand, 4,4′,4″-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT) (Tocris) at the dose of 10 mg/kg per day or vehicle diluted with 10% molecular-grade ethanol (EM Sciences) and 90% Miglylol 812N liquid oil (Sasol North America), beginning 7 d before adoptive transfer. This dose of PPT has been previously established (7). Animals were monitored daily for EAE signs based on a standard EAE 0 to 5 scale scoring system: 0, healthy; 1, complete loss of tail tonicity; 2, loss of righting reflex; 3, partial paralysis; 4, complete paralysis of one or both hind limbs; and 5, moribund.

Histological Preparation. Female mice were deeply anesthetized in isoflurane and perfused transcardially with ice-cold 1× PBS for 20 to 30 min, followed by 10% formalin for 10 to 15 min. Spinal cords were dissected and submerged in 10% formalin overnight at 4 °C, followed by 30% sucrose for 24 h. Spinal cords were cut in thirds and embedded in optimal cutting temperature compound (Tissue Tek) and frozen at −80 °C. The 40-μm thick free-floating spinal cord cross-sections were obtained with a microtome cryostat (model HM505E) at −20 °C. Tissues were collected serially and stored in 0.1 M PBS with 1% sodium azide in 4 °C until immunohistochemistry.

Immunohistochemistry. Before histological staining, 40-μm thick free-floating sections were thoroughly washed with 0.1 M PBS to remove residual sodium azide. For tissues to be treated with diaminobenzidine (DAB), sections were permeabilized with 0.5% Triton X-100 in 0.1 M TBS and 10% normal goat serum (NGS) for 60 min at room temperature. The following primary antibodies were used: anti-CD3 at 1:2,000 (BD Pharmigen), anti–neurofilament (NF200) at 1:750 dilutions (Sigma), anti–Iba-1 at 1:10,000 (Wako Chemicals), antiglial fibrillary acidic protein (GFAP) at 1:40,000 (Dako), anti-ERα at 1:10,000 (Millipore), anti-Neun at 1:750 (Sigma), and anti-myelin basic protein (MBP) at 1:750 (Sigma). Tissues were then washed three times for 10 min in 0.1 M TBS. Tissues were labeled with secondary antibodies conjugated to Cy5 (Vector Labs and Chemicon) for 1 h for NF-200, MBP, Neun, and GFAP. Tissues were labeled with biotin secondary antibodies for CD3 and Iba-1, followed by ABC/DAB treatment (Vector Labs). Fluorescent sections were mounted on slides, allowed to semidry, and coverslipped in fluoromount G (Fisher Scientific). DAB sections were dried overnight and then dehydrated in 70, 95, and 100% ethonal, followed by 5 min of Ctitrasolve and coverslipped with Permount (Fisher). IgG-control experiments were performed for all primary antibodies, and only nonimmunoreactive tissues under these conditions were analyzed. Immunohistochemistry for ERα was followed directly as previously described (8).

Quantification. To quantify the astrocyte culture PCR and Western blot results, we quantified the optical density from the gel bands using ImageJ Software v1.30, downloaded from the National Institutes of Health Web site (http://rsb.info.nih.gov/ij). Optical densities of WT and aCKO ERα bands were normalized to the positive control of ovarian tissue. To quantify immunohistochemical staining results, three spinal cord cross-sections at the T1 to T5 level or three hippocampal sections from each mouse were captured under microscope at 10× or 40× magnification using the DP70 Image software and a DP70 camera (both from Olympus). All images in each experimental set were captured under the same light intensity and exposure limits. Image analysis was performed using ImageJ Software v1.30. Three sections from each animal were then quantified to calculate the mean per animal. Immunohistochemical experiments were combined from three separate clinical trials. To control for variance, each immunohistochemical experiment was run as one large experiment with an $n = 9$–12 per group. Each immunohistochemical experiment was repeated at least twice to confirm data. Axonal densities were calculated by counting the number of $NF200^+$ cells in a 40× image over the area of the captured tissue section. Inflammatory infiltrates were quantified by counting the number of DAB-positive cells in the dorsal column of the thoracic spinal cord at 40× under a light microscope. Myelin (MBP) and GFAP were calculated as percent area intensity from the dorsal column.

Microscopy. Stained sections were examined and photographed using a confocal microscope (Leica TCS-SP) or a fluorescence microscope (BX51WI; Olympus) equipped with Plan Fluor objectives connected to a camera (DP70, Olympus). Digital images were collected and analyzed using Leica confocal and DP70 camera software. Images were assembled using Adobe Photoshop (Adobe Systems) and Microsoft PowerPoint. DAB sections were examined at the light level at 40× (Nikon Alphaphot-2 YS2).

Mononuclear Cell Isolation. To isolate mononuclear cells from the brain and spinal cord, animals were deeply anesthetized with isoflurane and perfused transcardially with ice-cold 1× PBS for 20 to 30 min. Brains were dissected and spinal cords were flushed with 1× PBS into complete RPMI medium (Lonza). CNS tissues were digested with Liberase Blendzyme I (Roche Applied Science), DNaseI (Invitrogen), and 1 mM $MgCl_2$ (Sigma) in HBSS for 30 min at 37 °C, then passed through a wire mesh screen, followed by 100-, 70-, and 40-μm nylon cell strainers to obtain single-cell suspensions. Cells were washed in complete RPMI medium and suspended in 50% Percoll (GE Healthcare Biosciences) medium in HBSS. Mononuclear cells were collected at the 63%/50% interface of a 63%/50%/30% Percoll step gradient following 30-min centrifugation at 1,800 rpm at 4 °C.

Flow Cytometry. Mouse mononuclear cells or splenocytes were collected on a 96 v-shaped plate (Titertek Co.) for flow cytometric analysis. Single-cell suspensions in FACs buffer (2% FCS in PBS) were incubated with anti-CD16/32 at 1:100 dilution for 20 min at 4 °C to block Fc receptors, centrifuged, and resuspended in FACs buffer with the following antibodies (Abs) added at 1:100 dilution for 30 min at 4 °C: anti-CD45, anti-CD3ε, anti-CD11b, anti-CD11c, and Rat-IgG2b, and Hamster-IgG isotype controls (Biolegend). Cells were subsequently washed twice in FACs buffer and then acquired on FACSCalibur (BD Biosciences), and analyzed by FlowJo software (Treestar). Quadrants were determined using cells labeled with appropriate isotype control Abs. Total cell number for each population identified by flow cytometry was determined by multiplying the percentage of positive cells by the global cell count as determined by Trypan blue staining and light microscopy. Statistical significance was determined by one-way ANOVA.

Statistical Analysis. Differences in EAE clinical scores were determined by repeated-measures one-way ANOVA. Immunohistochemical data were analyzed by one-way ANOVA. For these analyses, one-way ANOVA, Bonferroni post hoc analysis was performed on F-stat values and significance was determined at the 95% confidence interval (Prism).

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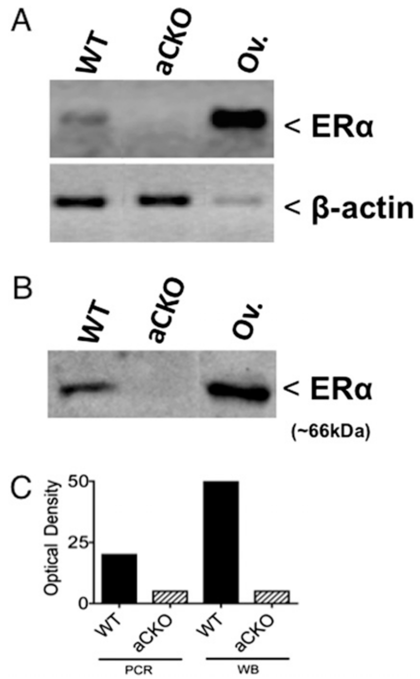

Fig. S1. RT-PCR and Western blot evidence that ERα is deleted from aCKO astrocytes. (*A*) RT-PCR and (*B*) Western blot show that ERα is essentially absent in primary astrocyte cultures from aCKO mice compared with WT mice. Ovary (OV) was used as a positive control. (*C*) Optical density of bands. $n = 4$ per group.

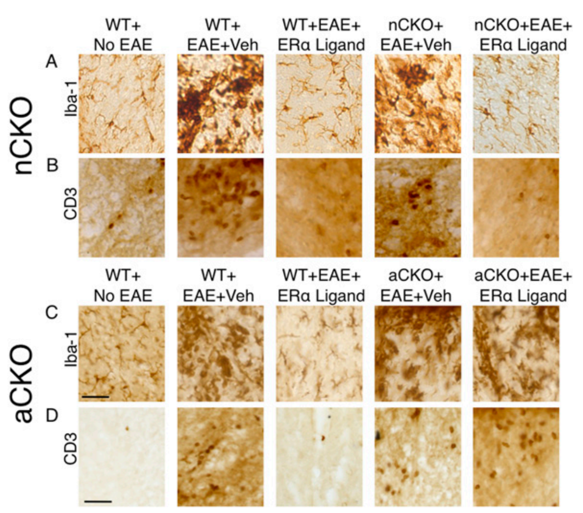

Fig. S2. Immunohistochemical evidence that ERα is required in astrocytes, but not neurons, to reduce Iba-1 globoid macrophage and CD3 T-cell inflammation in dorsal column white matter of thoracic spinal cord. (*A* and *B*) Iba-1 globoid macrophages and CD3 T cells are increased in WT mice and neuronal-ERα-CKO (nCKO) with EAE compared with WT mice without EAE. Treatment with ERα ligand ameliorates these increases in both WT mice and in nCKO mice with EAE. (*C* and *D*) Iba-1 globoid macrophages and CD3 T-cells are increased in WT mice and astrocyte-ERα-CKO (aCKO) with EAE compared with WT mice without EAE. Treatment with ERα ligand ameliorates these increases in WT mice but not in aCKO mice with EAE. (Scale bars, 25 μm.)

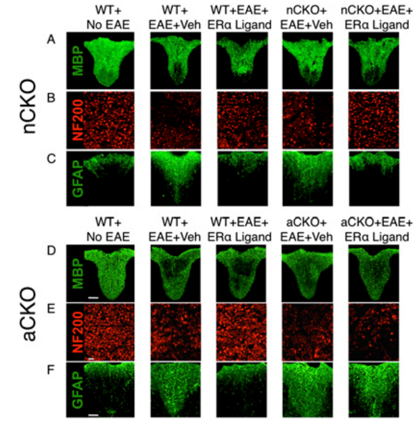

Fig. S3. Immunohistochemical evidence that ERα is required on astrocytes, but not neurons, to protect against axonal loss and gliosis in dorsal column white matter of thoracic spinal cord. (*A* and *D*) MBP exhibits a patchy reduction in all groups with EAE. (Scale bar, 60 μm.) (*B* and *E*) NF200^{+} axons exhibit patchy reductions in numbers in WT mice with EAE. Treatment with ERα ligand ameliorated axonal loss in WT and nCKO mice, but not aCKO mice, with EAE. (Scale bar, 30 μm.) (*C* and *F*) GFAP staining increased in intensity and area in WT mice with EAE. Treatment with ERα ligand ameliorated this increase in WT and nCKO mice, but not aCKO mice, with EAE. (Scale bar, 30 μm.)