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Estrogen Signaling through Estrogen Receptor Alpha in Astrocytes Mediates Neuroprotection during Experimental Autoimmune Encephalomyelitis and Decreases Astrocyte Levels of Proinflammatory Chemokines

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Cellular and Integrative Physiology

by

Rory Desmond Spence

2013

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ABSTRACT OF DISSERTATION

Estrogen Signaling through Estrogen Receptor Alpha in Astrocytes Mediates Neuroprotection during Experimental Autoimmune Enchephalomyelitis and Decreases Astrocyte Levels of Proinflammatory Chemokines

by

Rory Desmond Spence

Doctor of Philosophy in Molecular Cellular and Integrative Physiology

University of California, Los Angeles, 2013

Professor Rhonda R. Voskuhl, Chair

Estrogen has well documented neuroprotective effects in a variety of clinical and experimental disorders of the central nervous system (CNS). The beneficial effects of estrogens in CNS disorders include mitigation of clinical symptoms as well as attenuation of histopathological signs of neurodegeneration and inflammation. The cellular mechanisms that underlie these CNS effects of estrogens are uncertain, because a number of different cell types express estrogen receptors in the peripheral immune system and CNS. Here, we investigated the potential roles of two endogenous CNS cell types in estrogen-mediated neuroprotection. We selectively deleted estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ) from either neurons or astrocytes using well-characterized Cre-loxP systems for conditional gene knockout in mice and studied the effects of these conditional gene deletions in a well-characterized model of adoptive experimental autoimmune encephalomyelitis (EAE). We found that the pronounced and significant neuroprotective effects of systemic treatment with ERβ ligand on clinical function, CNS inflammation, and axonal loss during EAE were not completely prevented by conditional

deletion of ERβ from astrocytes or neurons. Interestingly, we found that the pronounced and significant neuroprotective effects of systemic treatment with ERα ligand on clinical function, CNS inflammation, and axonal loss during EAE were completely prevented by conditional deletion of ERα from astrocytes, whereas conditional deletion of ERα from neurons had no significant effect. Given the differential neuroprotective effects of ERα ligand treatment versus ERβ ligand treatment on astrocytes, as well on T-cell and macrophage inflammation, we looked for molecules within astrocytes that were affected by signaling through ERα, but not ERβ. We found that ERα ligand treatment, but not ERβ ligand treatment, decreased expression of the chemokines CCL2 and CCL7 by astrocytes in EAE. Together our data show that neuroprotection in EAE mediated via ERβ signaling does not require ERβ on astrocytes or neurons, whereas neuroprotection in EAE mediated via ERα signaling requires ERα on astrocytes, and not neurons, and reduces astrocyte expression of chemokines that contribute to CNS inflammation. These findings reveal important cellular differences in the neuroprotective mechanisms of estrogen signaling through ERα and ERβ in EAE.

The dissertation of Rory Desmond Spence is approved.

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CHAPTER I

An Introduction to Estrogens and Androgens as a Neuroprotective Treatment for Multiple Sclerosis and the Animal Model Experimental Autoimmune Encephalomyelitis

Multiple Sclerosis (MS)

Multiple Sclerosis (MS) is an autoimmune disease characterized by inflammation and demyelination in the CNS from unknown causes (Markovic-Plese & McFarland 2001; Trapp & Nave 2008). Clinical symptoms usually begin to occur in young adults. Nearly 80% of patients develop a relapse-remitting (RR-MS) course of disease, which can eventually become a more chronic secondary progressive (SP-MS) form after many years. About 15% of patients exhibit disease progression from the start called primary progressive MS (PP-MS) (Compston & Coles 2002). With myelin thought to be the primary target of the immune cells, areas of inflammation and demyelination constitute the MS lesions, with these lesions classically in white matter (Lassmann & Lucchinetti 2008). Lesions have been further subdivided into categories of active, chronic active and chronic inactive based on pathology (Lassmann & Lucchinetti 2008; Lucchinetti et al. 2000). Axonal transection and axonal loss can occur in lesioned areas perhaps contributing to permanent disability. In normal appearing white and grey matter pathology also exists as demonstrated by magnetic resonance imaging (MRI) and pathologic studies of post-mortem MS tissue (Herz et al. 2010).

MS is classically viewed as a CD4+ Th1 mediated immune disease. However, a host of immune and non-hematopoietic cells are also involved in the course of the disease including CD8+ T-cells, B-cells, macrophages, dendritic cells, astrocytes and oligodendrocytes to name a few (Markovic-Plese & McFarland 2001; McFarland & Martin 2007; Sospedra & Martin 2005). One major question in MS involves understanding whether immune inflammation is a primary cause or a secondary effect of neurodegeneration (Trapp & Nave 2008). Current MS treatments only treat the inflammatory component of the disease. However, neurodegeneration continues to occur even when inflammation is suppressed. Thus, while neurodegeneration may be triggered initially by inflammation, it appears that a neurodegenerative process may continue and eventually become relatively independent of inflammation. Therefore, there is a need for neuroprotective treatment in combination with an anti-inflammatory treatment to potentially achieve complete protection during MS (Meuth et al. 2010).

Experimental Autoimmune Encephalomyelitis (EAE)

EAE is the most common mouse model of MS (Croxford et al. 2011; R. Gold 2006). It is a CD4+ Th1/Th17 mediated autoimmune disease in which perivascular T-cells, followed by macrophages, enter the CNS, leading to lesioned areas of demyelination and axonal loss (Herz et al. 2010). This demyelination and axonal loss correlates with motor deficits in standard EAE clinical scores which primarily assess walking ability (Wujek et al. 2002). EAE has strain specific effects. In the SJL model, the disease is relapse-remitting, similar to that of RR-MS. In the C57BL/6 model, EAE follows a more progressive course, resembling PP-MS or SP-MS (Croxford et al. 2011). EAE may be either active or adoptive depending upon the method of induction (Miller & Karpus 2007). The basic concept of both active and adoptive EAE is to induce an immune response to a myelin antigen. In active EAE, an animal is immunized with a myelin antigen along with the nonspecific immune stimulators complete Freund's adjuvant (CFA) and tuberculosis bacterium (TB). Pertussis toxin (PTx) is also given in active EAE. The T-cells generated through this immunization regimen are able to cross the blood to brain barrier (BBB) and propagate an influx of monocytes into the CNS. They then alter the cytokine/ chemokine profile of the cells there in. Subsequent activation of resident microglia, and perhaps CNS cells such as astrocytes, simultaneously occurs as well. All of these steps lead to the demyelination of axons and axonal transection both within and beyond these areas of immune cell infiltration. Thus in active EAE, both the induction of the immune response and the neurodegenerative effects of the immune attack occur in the same mouse. In contrast, adoptive EAE separates the induction and the effector phase of EAE so that each phase can be studied separately. The induction phase is started in a donor mouse, in which the immunization is given over draining lymph nodes. The lymph nodes cells (LNCs) are then harvested and re-stimulated *ex-vivo* with the myelin antigen. These cultured T-cells and monocytes are then injected into a recipient animal to begin the effector phase of adoptive EAE. Immune infiltration then occurs in the CNS, followed by demyelination and axonal loss in the recipient mouse (Miller & Karpus

2007). Thus, the central difference between active and adoptive EAE is that in adoptive EAE the initiation of the immune response occurs in a different mouse, the donor, from in the one who exhibits disease, the recipient. This difference in the initiation of immune response between the two models can allow researchers to tease apart the immune response in EAE. For example, in adoptive EAE, one could treat a donor mouse with a compound, such as a hormone, and then inject the donor mouse's LNCs into an untreated recipient to understand how the hormone pre-treated LNC's might affect the CNS of a recipient mouse (Du, Sandoval, Trinh, Umeda, et al. 2010b). While the EAE model has its limitations, it has been the major preclinical model used to derive many current MS treatments (Croxford et al. 2011; Mix et al. 2010; Slavin et al. 2010).

Gender Differences in MS and EAE

There are large gender differences in the prevalence of human autoimmune diseases. Most of these gender differences entail a higher incidence in females as compared to males: systemic lupus erythematosis (SLE), rheumatoid arthritis (RA), Graves disease, and multiple sclerosis (MS) (Lockshin 2006; Martocchia et al. 2011; Beeson 1994; Fairweather et al. 2008; McCombe et al. 2009; Noonan et al. 2002; Bossert 2005; Oliver & Silman 2009; Sekigawa et al. 2010). While the reasons for these gender differences are still unknown, possible explanations include differences in sex hormones or sex chromosomes. Specifically, possibilities include: 1) a detrimental effect of endogenous ovarian hormones such as estrogens and/or progesterone in females 2) a protective effect of endogenous testicular hormones such as androgens in males, or 3) a detrimental effect of the XX sex chromosome compliment in the females, or 4) a protective effect of the XY sex chromosome compliment in males (Teuscher et al. 2010; Voskuhl 2011; Ngun et al. 2011). The EAE model has been useful to study these four non-mutually exclusive possibilities.

Similar to MS, sex differences exist in the EAE model. In the SJL, ASW, and NZW strains, female mice are more susceptible to EAE than males. However, in the B10.PL and PL/J

strains, male mice are more susceptible to EAE than females (Papenfuss et al. 2004). Ovariectomy of SJL female mice is controversial, with some groups showing no effect on disease and others showing some worsening of EAE scores (Voskuhl & Palaszynski 2001; Jansson et al. 1994; McClain et al. 2007). Together, these results suggest that endogenous estrogens are clearly not detrimental to EAE in females, but if anything may be protective. On the male side, castration of mice worsens EAE in the SJL strain, suggesting that endogenous androgens are indeed protective to males (Bebo et al. 1998; Voskuhl & Palaszynski 2001). It is important to keep in mind that while most sex hormones come from the gonads, the CNS, particularly during EAE, is capable of providing endogenous estrogens and androgens (Caruso et al. 2010). However, whether these levels reach physiological relevance remains unclear.

Differences in sex chromosomes also may contribute to sex differences in disease. By using mice which differed in sex chromosome complement while sharing a common gonadal phenotype (Arnold & Chen 2009) it has been shown in the SJL strain that the XX sex chromosome compliment confers increased severity of EAE as compared to the XY sex chromosome compliment. This suggested that either the XX complement is disease promoting or the XY complement is protective (Palaszynski et al. 2004; Smith-Bouvier et al. 2008). Other studies have shown that a Y chromosome-linked polymorphism can influence disease (Teuscher et al. 2006). Specifically, EAE was more severe in young male consomic mice carrying a B10.S Y chromosome as compared to age and sex matched SJL mice. The authors therefore concluded that a polymorphism on the Y chromosome of SJL mice contributes to the well known observation that young male mice exhibit temporary protection from disease (Spach et al. 2009). It remains unclear as to why a polymorphism would be protective in younger, but not older, mice. Together the data would suggest an interaction between sex chromosome genes and other factors related to aging, with these other factors theoretically being either hormonal or nonhormonal. One possible nonhormonal factor, regardless of age, could be genomic imprinting. Previous research has suggested that genomic imprinting such as histone modifications, methylation, and endogenous miRNA could possibly play a role in autoimmunity

and sex differences (Camprubí & Monk 2011; Gabory et al. 2009). In summary, while many assume that the sex bias in MS is due to sex hormones, one must appreciate that sex chromosome genes may also play a role.

Currently, there is an extensive amount of literature highlighting the effect of estrogens and androgens in EAE and MS (El-Etr et al. 2011; Offner 2004; Offner & POLANCZYK 2006; Voskuhl & Palaszynski 2001). In this review we will build upon this existing literature by focusing on specific sex hormone receptors and how they contribute to disease protection during EAE and MS.

Pregnancy in MS and EAE

In MS, during the last trimester of pregnancy, circulating levels of estrogens are at their highest levels correlating with a reduction in relapse rates among women with MS. Post-partum levels of estrogens drop precipitously and correlate with a significant increase in relapse rates during the 3-6 months after delivery (Confavreux et al. 1998). While controversial, some reports have demonstrated that pregnancy could offer long term protection to women with MS (Runmarker & Andersen 1995; Verdru et al. 1994). MS patients who had one or more pregnancies were wheelchair dependent after 18.6 years, versus 12.5 years for the control group (Verdru et al. 1994). Others have shown that pregnancy can regulate the immune response in MS patients. Peripheral blood lymphocytes from pregnant women with MS were mitogen stimulated to examine the Th1 and Th2 cytokine profile during pregnancy and post-partum. The patients showed a remarkable shift from Th2 profile during pregnancy to a Th1 cytokine profile post-partum, further providing evidence of pregnancy's effect in this Th1 mediated disease (Al-Shammri et al. 2004)[1].

Pregnancy also offers protection to female animal models with EAE (Gatson et al. 2011; Brenner et al. 1991; Evron et al. 1984; Korn-Lubetzki et al. 1984; McClain et al. 2007). Specifically, pregnant SJL and C57BL/6 mice showed a disease amelioration compared to non-pregnant controls. Further, lymphocytes from pregnant EAE mice showed a decrease in TNFα

and IL-17 with an increase in IL-10 (McClain et al. 2007). Given the powerful effect of late pregnancy on EAE and MS, and since estrogens are at high levels during late pregnancy, studies have examined if exogenous estrogens are capable of providing disease protection in EAE and MS.

Estrogens and Neuroprotection

Estrogens are made up of three endogenous biologically different compounds: estrone, estradiol, and estriol (Ghayee & Auchus 2007; Zhu & Conney 1998). Furthermore, exogenous selective estrogen receptor modulators (SERMS) are capable of activating either estrogen receptor alpha (ERα) or estrogen receptor beta (ERβ) on select tissues (Lewis & Jordan 2005). Testosterone and dihydrotestosterone (DHT) are able to act on the androgen receptor (AR). However, DHT is also able to act through ERβ (Kuiper 1997; Lund et al. 2006). It is always difficult to decipher the action of testosterone since it can be aromatized to estrogens through the enzyme aromatase. Hence, either the non-aromatizable 5αDHT, or AR antagonists can be used to study the effect of androgens on actions of ARs (Ghayee & Auchus 2007; Kemppainen et al. 1999).

Treatments with estrogens have demonstrated diverse mechanisms of possible neuroprotection in a number of diseases, including Parkinson's disease (PD), Alzheimer's disease (AZ), ischemic stroke, spinal cord injury and MS (Garcia-Segura et al. 2001; Brann et al. 2007; Samantaray et al. 2010; Suzuki et al. 2009; Cho et al. 2003). In clinical studies, women who took estrogen replacement therapy were at less risk of developing PD (Currie et al. 2004). Woman who received short-term estrogen treatment had an increase in dopamine transporters in the caudate putamen (Gardiner et al. 2004). Multiple groups have shown that in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) animal model of PD, exogenous physiological levels of estradiol were able to protect against loss of DA neurons (Kenchappa 2004; Ramirez et al. 2003; Shughrue 2004). Others have shown that the in PD animal model, 6-hydroxdopamine (6-OHDA), estradiol can also act indirectly by activating the insulin-like growth factor-1 (IGF-1)

receptor to protect against 6-OHDA induced neuronal loss (Quesada & Micevych 2004).

In AD patients, estrogen treatment decreased the risk of disease, particularly in younger (50-62yo) patients, but was not effective in older groups of patients (Henderson et al. 2005). This early protection during AD could be due to estradiol's ability to increase amyloid beta-protein (Aβ) uptake by microglia as well as prevent Aβ peptide formation by neurons (Li et al. 2000; Xu et al. 1998). It is known that in spinal cord injury, estrogen can protect against neuronal loss. Data suggest that this prevention of neuronal loss could be due to estradiol's ability to prevent the actions of Ca^{2+} activated proteases (Samantaray et al. 2010; Ray et al. 2011). Furthermore, others have shown that estradiol was able to reduce neuronal death and loss of lesion volume in ischemic stroke (Suzuki et al. 2009; Gibson et al. 2006).

Estrogens can also act on other cells in the diseased CNS besides neurons. For instance, estrogen increases astrocytes ability to uptake glutamate to prevent neuronal loss due to glutamate toxicity (Arevalo et al. 2010; Dhandapani & Brann 2007). Others have shown that estrogen can increase transforming growth factor beta (TGFβ-1) and TGFβ-2 from astrocytes (Dhandapani 2005). Estrogens also have a suppressing effect on neuroinflammation (Vegeto et al. 2008). Estradiol strongly inhibits microglia activation by lipopolysaccharide (LPS) (Drew & Chavis 2000; Vegeto et al. 2001). In addition, estradiol can inhibit intracellular localization of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB), which can prevent downstream activation of a variety of inflammatory genes (Ghisletti et al. 2005).

With estrogen's ability to provide protection in a number of CNS disease models, much research has been focused on the mitochondria as a target of estrogens actions. Treatments with estrogens have a direct neuroprotective effect on mitochondria (Brinton 2008a; Irwin et al. 2008; Nilsen et al. 2007; Nilsen & Brinton 2004). Estrogens have been shown to increase aerobic glycolysis, respiratory efficiency, ATP generation, $Ca2+$ load tolerance and act as an antioxidant defense (Brinton 2008b). Furthermore, estrogens can alter pro and anti-apoptotic molecules that act on the mitochondria. For instance, estrogen can increase anti-apoptotic markers, bcl-2 and bcl-xl, while decreasing the pro-apoptotic marker bax in animal models of AD

(Nilsen et al. 2007; Nilsen & Brinton 2004). Estrogen's antioxidant properties also allow it to protect against reactive oxygen species from the mitochondria (Razmara et al. 2007). It is important to note that the timing of estrogen administration is critical to ensure estrogens protective outcome as noted in the *healthy cell bias of estrogen action hypothesis*. If estrogens are administered too late in the disease they are not protective (Brinton 2008b; Nilsen 2008).

Estradiol and EAE

Treatment with a wide dosage range of estradiol is able to ameliorate EAE during both the induction and effector phases of both active and adoptive EAE. This amelioration can occur in both sexes of mice, as well as multiple mouse strains with EAE (Elloso 2005; Voskuhl & Palaszynski 2001; Bebo et al. 2001; Jansson et al. 1994; Matejuk et al. 2001; Offner et al. 2000; Subramanian et al. 2003). The protective effects of estradiol in EAE include a decrease in inflammatory cells in the CNS as well as protection against demyelination (Offner 2004) (Bebo et al. 2001; Subramanian et al. 2003). With respect to inflammation, estradiol treatment was able to alter cytokine production as well as alter chemokine/chemokine receptors, growth factors, and adhesion molecules in EAE mice. Specifically, estradiol treatment was able to decrease chemokine (C-C motif) ligands and receptors, tumor necrosis factor-alpha (TNF-α), and others, while increasing transforming growth factor beta (TGFβ)-2 and TGFβ-3 mRNA in the spinal cord of EAE mice (Matejuk et al. 2001; Matejuk et al. 2003; Subramanian et al. 2003) (Table 1). Further studies have shown that mononuclear cells isolated from the brain of estradiol treated EAE mice show a decrease in TNFα, IFNγ, and others (Bodhankar et al. 2011; Subramanian et al. 2003) (Table 1). In splenocytes and/or peripheral LNCs, estradiol was able to decrease TNFα, neural cell adhesion molecule (NCAM) and others while increasing cytotoxic T-lymphocyte antigen-4 (CTLA-4), vascular cell adhesion molecule (VCAM) and more (Bodhankar et al. 2011; Lelu et al. 2011) (Matejuk et al. 2001; Matejuk et al. 2002; Subramanian et al. 2003) (Table 1).

Dendritic cells appear to be one potential target of estradiol's protective actions during

EAE. Dendritic cells are potent antigen presenting cells and are thought to play a role in T-cell activation in EAE (Almolda et al. 2011). Estradiol treatment resulted in a decrease in activated dendritic cells in the CNS and spleens of EAE mice. Mature dendritic cells were shown to have decreased expression of TNFα, IFNγ, and IL-12 mRNA with estradiol treatment (Table 1). Finally, T-cells cocultured with dendritic cells that were pretreated with estradiol showed a shift from Th1 to Th2 cytokine production (H. Y. Liu et al. 2002) Further research has also shown that dendritic cells are a target of estradiol in Lewis rats with EAE. Rats injected with dendritic cells that were pretreated with estradiol showed a decrease in CD4+ T-cells and CD68+ macrophages in the CNS compared to naïve injected dendritic cells (Pettersson et al. 2004).

Others have focused their research on T-cells as the target cell of estradiol's protective effect (Offner et al. 2005; Polanczyk, Jones, et al. 2010c; Offner et al. 2000). Studies have shown that the decrease in T-cells into the CNS of estradiol treated mice was not due to a decrease in T-cell proliferation, but in fact, the ability of T-cells to enter the CNS (Bebo et al. 2001; Subramanian et al. 2003). Although estradiol might not directly act on T-cells during EAE to provide disease protection, estradiol appears to indirectly alter the sub-populations of T-cells (Offner et al. 2005). For example, matrix metallopeptidase-9 (MMP-9), has been shown to allow T-cells to migrate across the BBB. Research has shown that estradiol was able to decrease MMP-9 expression on T-cells during EAE (S. M. Gold et al. 2009), perhaps explaining why T-cell numbers are decreased in the CNS of estradiol treated EAE mice (Subramanian et al. 2003). Furthermore, estradiol was able to upregulate FoxP3, contributing to a protective effect from CD4+CD25+ regulatory T-cells (Offner 2004; Offner & POLANCZYK 2006; Polanczyk et al. 2005). However, removal of Foxp3+ T-cells did not appear to alter estradiol's protective effect in EAE mice, suggesting other cells, such as B-cells or CNS cells might be responsible for estradiol's protection (Subramanian et al. 2010).

B-cells also appear to be indispensible in responding to estradiol's protective effects during EAE (Subramanian et al. 2010). Removal of B-cells from EAE mice abrogates the protective effects of estradiol (Bodhankar et al. 2011). When the B-cells were examined from

estradiol treated mice, there was an upregulation of programmed cell death ligand-1 (PD-L1) as well as an increase in IL-10 (Bodhankar et al. 2011). Further research is needed to fully understand estradiol's actions on B-cells in EAE.

Estrogen Receptor Alpha Ligand and EAE

Given that estradiol is able to act both on ERα and ERβ, a host of research has now been focused on which receptor might be responsible for the protective effects of estrogens during EAE. While data have shown that this effect is mediated through ERα (Lélu et al. 2010; Lelu et al. 2011; Polanczyk, Zamora, et al. 2010b), other data have shown that ERβ can also offer protection during EAE (Crawford et al. 2010; Du, Sandoval, Trinh, Umeda, et al. 2010b).

Originally it was shown that EAE was less severe in B10.PL male mice with a global knockout of the ERα gene, termed Esr1 (-/-), as compared to WT, Esr1 (+/+) male mice, suggesting that endogenous estrogens are disease promoting during EAE (Polanczyk, Yellayi, et al. 2010a). The source of estrogens in these male mice was unclear, but in retrospect it could have been from conversion of testostosterone to estradiol *in vivo* by aromatase or from injury induced upregulation of estradiol production locally within the CNS. Later data from the same group, as well as another, each treated wild type and global ERα knock out mice with higher than peak ovulatory doses of either estradiol or estriol, respectively, to ascertain whether these exogenously administered estrogens worked through ERα to ameliorate EAE (Polanczyk, Zamora, et al. 2010b; H.-B. Liu et al. 2003). While these two papers each showed a critical role for ERα in disease protection mediated by exogenous estradiol or estriol treatment, there were no differences in disease between placebo treated global ERα knock outs and wild types in either females or males. Notably, as discussed elsewhere, the effect of ovariectomy on EAE has been controversial, with some groups finding no significant consistent effect of ovariectomy on EAE (Voskuhl & Palaszynski 2001) and others finding a worsening of EAE thereby suggesting a disease protective effect of endogenous estrogens (Jansson et al. 1994). Together, the lack of an effect of the global ERα knock out on placebo treated mice with EAE is consistent with the

lack of an effect of ovariectomy on disease.

Exogenous ERα ligand, propyl pyrazole triol (PPT), treatment provides disease protection in a variety of mouse strains with EAE (Elloso 2005; S. M. Gold et al. 2009; Morales 2006; Spence et al. 2011; Tiwari-Woodruff et al. 2007). In active EAE, pre-treatment with the ERα ligand abolishes clinical disease as evidenced by the loss of this protection in different ERα KO mouse models (Morales 2006; Polanczyk, Zamora, et al. 2010b). Furthermore, in the effector phase of EAE, ERα ligand treatment provides clinical disease protection compared to EAE with vehicle control treatment. Although the disease was not completely abolished with treatment only in the effector phase, the peak disease score was lower and the overall severity of the disease was significantly reduced (Spence et al. 2011).

In the peripheral immune system during active EAE, ERα ligand treatment was able to decrease pro-inflammatory cytokines such as TNFα, IFNγ, and more while increasing the anti-inflammatory cytokine IL-5 in cultured splenocytes and LNCs (Elloso 2005; Morales 2006; Tiwari-Woodruff et al. 2007)(Table 1). Within the CNS, ERα ligand treatment was capable of decreasing macrophage and T-cell inflammation at both early and late time points of EAE (Morales 2006; Tiwari-Woodruff et al. 2007). Beyond inflammation, ERα ligand treatment has been shown to protect against axonal loss as well as demyelination at both early and late time points of EAE (Morales 2006; Tiwari-Woodruff et al. 2007). While the mechanism of this protection isn't fully understood, recent studies have shown that ERα ligand treated mice with EAE had reduced MMP-9 in supernatants from auto-antigen stimulated splenocytes. This reduction in MMP-9 coincided with a decrease in T-cell and macrophage infiltration into the CNS (S. M. Gold et al. 2009) (Table 1).

Given the powerful effects of ERα ligand treatment on EAE, focus shifted toward what cell or host of cells were responsible for ERα ligand's protective actions. ERα is expressed on a variety of cells including but not limited to: T-cells, macrophages, microglia, neurons, oligodendrocytes, astrocytes, and epithelial cells (Danel et al. 1985; Garc a-Ovejero et al. 2002; Garidou et al. 2004; Mitra 2003; Pérez et al. 2003; Vegeto et al. 2001; Gulshan et al. 1990). It

was assumed that ERα ligand was acting on peripheral immune cells to prevent an immune response in EAE, thus ameliorating disease. However, bone marrow chimera studies and selective ERα KO studies, in which ERα was selectively removed from T and/or B-cells, demonstrated that peripheral T and B-cells were not the direct target cell of estrogen mediated protection (Garidou et al. 2004). However, a recent study used bone marrow chimeras to generate a mouse that was deficient in ERα on hematopoietic cells. While there was no effect on the clinical course of EAE, the mice that lacked ERα on hematopoietic cells had a larger amount of CD4+ T-cell infiltration into the CNS, suggesting that ERα expression on immune cells was involved in reducing CNS infiltration (Lélu et al. 2010). A new study has now shown that T-cells may indeed be a direct target of ERα ligand's protective effects depending upon the timing and dosage of exogenous ERα ligand treatment (Lelu et al. 2011). Furthermore, B-cells also appear to be a target cell of interest. Estradiol treated B-cells that were removed from ERα -/- mice were unable to suppress a proliferative response from co-cultured T-cells when compared to B-cells from WT mice, thus demonstrating that estradiol acted through ERα on B-cells to inhibit an immune response (Bodhankar et al. 2011). Clearly, more research is necessary to determine if T and/or B-cells are the direct or indirect targets of ERα ligand treatment *in vivo* during EAE.

Regarding potential cellular targets in the CNS, astrocytes are known to express ER's (Garc a-Ovejero et al. 2002). Removal of reactive astrocytes was shown to worsen EAE. Specifically, clinical disease, as well as levels of CNS inflammation and axonal loss, were increased in EAE animals that had transgenic ablation of reactive astrocytes compared to WT mice with EAE (Voskuhl et al. 2009). Thus, a conditional knock out, whereby ERα was removed only in astrocytes, was created. When EAE was induced in this conditional knockout of ERα from astrocytes, ERα ligand treatment was no longer protective (Spence et al. 2011). We will go more detail into astrocytes and estrogens in the following chapters. While ERα expression on astrocytes plays an important role in the protective effect of ERα ligand treatment, further

research is needed to determine if there are other cellular targets in the CNS that may mediate ERα ligand’s neuroprotective effects.

Estrogen Receptor Beta Ligand and EAE

Data have shown that the global knock out of the ERβ gene does not alter EAE disease severity compared to WT mice, suggesting endogenous estrogens do not act through ERβ to offer disease protection (Polanczyk, Yellayi, et al. 2010a; Polanczyk, Zamora, et al. 2010b). However, exogenous treatment with an ERβ ligand has been shown to ameliorate EAE in the C57BL/6 strain, but not in the SJL strain (Elloso 2005; Tiwari-Woodruff et al. 2007). This difference in clinical protection could be due to strain differences or due to differences in the timing of the effect of treatment on disease (Du, Sandoval, Trinh & Voskuhl 2010a; Elloso 2005; S. M. Gold et al. 2009; Tiwari-Woodruff et al. 2007). Our group has shown that EAE mice pre-treated with ERβ ligand exhibit lower disease scores than mice treated with vehicle. Although the disease was not completely abolished, clinical scores were significantly lower later in disease (Tiwari-Woodruff et al. 2007). In contrast to treatment with ERα ligand, ERβ ligand treatment did not affect cytokine production during peripheral auto-antigen specific immune responses. In addition, ERβ ligand treatment did not reduce spinal cord inflammation during EAE as assessed by immunohistochemistry (IHC) of the spinal cord. Nevertheless, treatment with ERβ ligand was still able to protect mice against demyelination and axonal loss. Further, ERβ ligand treatment was able to restore motor function as measured by the rotor-rod test in EAE mice (Tiwari-Woodruff et al. 2007). These results suggested that ERβ, while clinically beneficial, did not reduce levels of CNS inflammation and therefore might act directly on CNS cells to mediate neuroprotection.

Given ERβ ligand’s potential as a neuroprotective treatment for MS, our group studied the combination of ERβ ligand treatment with an anti-inflammatory agent currently used in MS, Beta-interferon. These studies in EAE confirmed that ERβ ligand treatment alone was effective at reducing clinical disease, as well as protecting against axonal loss even in the presence of

inflammation (Du, Sandoval, Trinh & Voskuhl 2010a). However, the combination of ERβ ligand treatment with Beta-interferon was more effective at ameliorating EAE than ERβ ligand treatment alone. Combination treatment significantly reduced clinical disease, preserved axonal density, and reduced inflammation. Specifically, combination treatment reduced T-cells and macrophages in the spinal cord, while decreasing IL-17 from splenocytes. In addition, the cell adhesion molecule, VLA4, was significantly reduced on T-cells with the combination treatment (Du, Sandoval, Trinh & Voskuhl 2010a). These data suggested that ERβ ligand treatment might be useful in combination with an anti-inflammatory agent in the treatment of MS.

Recent evidence has shown that ERβ ligand treatment was not only capable of preventing demyelination, but also has the ability to stimulate endogenous myelination (Crawford et al. 2010). In the corpus callosum, similar to previous observations in the spinal cord, ERβ ligand treatment prevented demyelination of callosum axons even in the presence of inflammation (Crawford et al. 2010; Tiwari-Woodruff et al. 2007). Furthermore, there was an increase in the number of mature oligodendrocytes that correlated with an increase in myelin sheath thickness with a corresponding decrease in the g-ratio. Electrophysiology recordings showed that callosum axon conduction, with ERβ ligand treatment, exhibited a significant increase in compound action potential amplitudes, latency, and axon refractoriness (Crawford et al. 2010). It remains unknown whether effects on myelination are due to direct effects of ERβ ligand on oligodendrocytes or indirect effects on other cells expressing ERβ.

While previous studies showed that ERβ ligand treatment does not affect the levels of inflammation in the CNS (Tiwari-Woodruff et al. 2007), subsequent studies asked whether there might be qualitative differences in similar levels of spinal cord inflammation in ERβ ligand versus vehicle treated EAE mice (Du, Sandoval, Trinh, Umeda, et al. 2010b). No differences were observed in the numbers of T-cells or macrophages comprising infiltration, but the numbers of dendritic cells in the CNS were reduced in ERβ ligand treated mice. Furthermore, ERβ ligand treatment decreased the amount of TNFα produced by these CNS dendritic cells (Table 1). Finally, adoptive EAE was induced in WT recipients using draining LNCs composed of dendritic

cells from either ERβ KO or WT mice. Upon treatment with ERβ ligand, the immune cells composed of ERβ deficient dendritic cells were no longer sensitive to ERβ ligand mediated immune suppression (Du, Sandoval, Trinh, Umeda, et al. 2010b). Furthermore, others have shown that ERβ ligand treatment can suppress inflammatory responses of microglia and astrocytes (Saijo et al. 2011). In summary, ERβ ligand differs substantially from ERα ligand in its effects on EAE, but both remain potential candidates for the treatment of MS.

GPR30 and EAE

While ERα and ERβ are classically viewed as nuclear receptors, G protein-coupled receptor 30 (GPR30) is expressed on cellular membranes of both human and mouse cells (Maggiolini & Picard 2010; Mizukami 2010; Prossnitz et al. 2008). Recent evidence suggested that estradiol binding to the GPR30 may contribute to some of the protective effects of estradiol treatment in EAE (Blasko et al. 2009; Wang et al. 2009; Yates et al. 2010). While not lost completely, the magnitude of the estradiol mediated protection during disease, specifically inflammation and demyelination, was reduced in GPR30KO mice on a C57BL/6 background (Wang et al. 2009; Yates et al. 2010). Furthermore, SJL and C57BL/6 mice treated with the GPR30 agonist, G-1, showed a small but significant decrease in clinical disease severity compared to control mice in both active and adoptive EAE (Blasko et al. 2009; Wang et al. 2009). G-1 treatment was also able to decrease axonal damage, CNS inflammation, and demyelination (Wang et al. 2009). Furthermore, there was a decrease in the number of macrophages in the CNS with G-1 treatment (Blasko et al. 2009). Cytokine and chemokine expression was also altered with G-1 treatment; specifically there was a decrease in IFNγ, TNFα, IL-17, IL-23 CCL2, CCL4 and CCL5 from cultured splenocytes and LNCs (Blasko et al. 2009). Additionally, G-1 treatment increased PD-1 in CD4+ Foxp3+ Treg cells, (Wang et al. 2009). While G-1 treatment was able to provide some level of protection during EAE, the exact mechanism of action remains unclear.

Estriol Treatment in EAE

Estriol can bind to both ERα and ERβ, but has a preference for binding ERβ over ERα (Kuiper 1997). Studies have shown that doses of estriol, consistent with physiological murine pregnancy levels, can ameliorate EAE in female mice (Bebo et al. 2001; Kim et al. 1999). Estriol treatment was protective in multiple strains of mice including SJL, C57/B6, and B10.PI. Furthermore, both female and male of SJL and C57BL/6 mice respond to estriol treatment, demonstrating that estriol treatment was not female specific (Bebo et al. 2001; S. M. Gold et al. 2009; Jansson et al. 1994; Kim et al. 1999; Offner et al. 2000; Palaszynski 2004).

Estriol treatment was able to prevent spinal cord inflammation and demyelination in EAE mice (Bebo et al. 2001; Kim et al. 1999). Multiple studies have shown that estriol treatment alters the cytokine profile to induce a shift from a Th1 to a Th2 from cultured splenocytes and LNCs. This shift included a decrease in TNFα, IFNγ, IL-2, IL-6, and an increase in IL-5 and IL-10 (Bebo et al. 2001; Kim et al. 1999; Palaszynski 2004) (Table 1). Others have shown that splenocytes from estriol treated mice exhibited a decrease in the migration marker MMP-9, consistent with the decrease in CNS inflammation in estriol treated mice (S. M. Gold et al. 2009). This reduction in MMP-9 was also found with ERα ligand treatment, but not with ERβ ligand treatment (S. M. Gold et al. 2009).

Recent evidence shows that dendritic cells, at least in part, mediate estriol's protective effect. Estriol treated dendritic cells exhibited a decrease in cytokines: IL-IL-6, IL-12 and more with an increase in the immunoregulatory cytokines: TGF-β and IL-10 (Papenfuss et al. 2011) (Table 1). Furthermore, activation markers, MHC II, CD80 and CD86, as well as inhibitory markers, PD-L1, PD-L2, B7-H3, and B7-H4 were all increased in estriol treated dendritic cells (Papenfuss et al. 2011). These estriol induced changes resulted in a decreased severity of EAE (Papenfuss et al. 2011). This evidence in the preclinical model lends support to exploration of estriol as a potential treatment for MS patients.

Estriol Treatment in MS

A pilot clinical trial of estriol was conducted in females with relapsing-remitting MS (RRMS). In the study, patients were observed for 6 months, then treated with pregnancy levels of oral estriol (8mg per day) for the following 6 months. After 6 months of treatment the patients were taken off estriol for 6 months and then placed back on treatment for another 4 months. During treatment, RRMS patients exhibited a significant decrease in the number of gadolinium enhancing lesions on brain MRI as compared to pretreatment baseline. When treatment stopped, enhancing lesions returned to baseline levels, and when treatment was again reinstated, enhancing lesions again decreased. Furthermore, cognitive function, using the paced auditory serial addition test (PASAT), was improved in RRMS patients, although this may have been confounded by practice effects in this single arm cross over study (Sicotte et al. 2007).

Estriol treatment was also able to beneficially alter the immune response in MS patients. For example, a decrease in the recall response to a control antigen, namely the delayed type hypersensitivity (DHT) response to tetanus, was observed (Sicotte et al. 2007). Furthermore, during the trial, PBMCS were collected longitudinally and then stimulated *ex vivo* with mitogens and recall Ags. PBMCs collected during the *in vivo* estriol treatment period showed a significant increase in IL-5 and IL-10, with a significant decrease in TNFα, IFNγ, and MMP-9 (S. M. Gold et al. 2009; Sicotte et al. 2002; Soldan et al. 2003)(Table 1). This Th1 to Th2 cytokine shift correlated with the decrease in enhancing lesions in RRMS patients. Data indicated that the increase in IL-5 was due to an increase in CD4+ and CD8+ T-cells, while the increase in IL-10 was due to an increase CD64+ monocytes/macrophages. The decrease in TNFα was due to a decrease in CD8+ T-cells (Soldan et al. 2003). Further exploration of estriol as a treatment in MS is warranted and ongoing (http://www.clinicaltrials.gov/ct2/show/NCT00451204).

Testosterone, 5αDHT, and EAE

SJL mice show a sex bias similar to humans with MS characterized by greater susceptibility in females as compared to males (Dalal et al. 1997). It appears that this sex bias is due at least in part to a protective effect of androgens. Castrated SJL male mice show a worsening of clinical disease as well as greater demyelination and inflammation in the spinal cord when compared to sham operated males (Bebo et al. 1998; Palaszynski et al. 2004). Interestingly, C57BL/6 mice show neither a sex difference in EAE nor an effect from male castration, suggesting that endogenous androgens are not protective in the C57BL/6 strain. Strangely, this effect is not due to strain differences in endogenous levels of testosterone. In contrast to endogenous testosterone, exogenous testosterone treatment ameliorated EAE in both the SJL and C57Bl/6 strains (Palaszynski et al. 2004; Bebo et al. 1999). Interestingly, supplemental testosterone did not provide disease protection in castrated middle-aged male C57BL/6 mice (Matejuk et al. 2005). It remains unclear regarding why endogenous testosterone is effective in the SJL strain but not the C57BL/6 strain; furthermore, it's unclear why exogenous testosterone is more beneficial in younger mice. Theoretically these differences in the effectiveness of testosterone on disease could be due to strain or age differences in AR or ER expression.

Testosterone has the ability to be aromatized into estrogen by the enzyme aromatase. Thus, testosterone may either work through ARs, ERs, or GPR-30 (Ghayee & Auchus 2007; Mizukami 2010; Thomas et al. 2010). To circumvent the effects of testosterone treatment on estrogen receptors, groups have used 5αDHT, the non-aromatizable androgen. 5αDHT was able to lessen disease severity SJL and C57BL/6 mice, suggesting that at least some of the protective effect of testosterone treatment could be mediated through the AR (Dalal et al. 1997; Palaszynski et al. 2004). Direct evidence of androgens working through the AR, comes from data showing that testosterone treatment was not able to completely ameliorate disease in the presence of the AR antogonist flutamide in C57BL/6 young male mice (Matejuk et al. 2005).

Androgens alter the immune profile of EAE mice. Castration of male SJL mice leads to an increase in immune cell infiltration into the CNS (Bebo et al. 1998). Evidence has shown that T-cells treated with 5αDHT produced more IL-10 than vehicle treated T-cells (Dalal et al. 1997), suggesting that some anti-inflammatory effects are mediated through the AR. In castrated SJL EAE mice, IFNγ levels were increased in whole spinal cord (Bebo et al. 1998)(Table 1). SJL female T-cells treated with testosterone, from either the spleen or lymph nodes, also showed a decrease in IFNγ with an increase in IL-10 (Bebo et al. 1999). Further evidence has shown that testosterone treatment decreased TNFα in splenocytes in male mice (Matejuk et al. 2005)(Table 1). Perhaps of most importance, SJL mice showed a decrease in adoptive EAE clinical disease when injected with T-cells pre-treated with testosterone versus untreated T-cells (Bebo et al. 1999). All of this evidence suggests that the androgens may provide some disease protection at least in part through the AR. Further studies are needed to determine what cells might be responsible for this protection.

Testosterone and MS

Given that males are less susceptible than females to MS, as well as the animal data demonstrating testosterone's beneficial effect in EAE, a preliminary study was performed in which men with MS were treated with testosterone. Ten men with RRMS were given a daily treatment of 10g of a gel containing 100mg of testosterone for 12 months. This was preceded by a 6-month observation period. Testosterone treatment resulted in a significant slowing of the rate of brain atrophy as measured by monthly MRI. Furthermore, the patients exhibited an improvement in cognitive testing. However, this effect could have been confounded by practice effects in this single arm cross over trial design (Sicotte et al. 2007). PBMCs obtained during the treatment period, as compared to the pretreatment period, produced significantly more brain-derived neurotrophic factor (BDNF), platelet derived growth factor (PDGF)-BB, and TGFβ-1 with decreased IL-2 production (Table 1). Furthermore, testosterone treatment reduced the DTH recall immune response as well as decreased CD4+ T-cells and increased NK cells (S. M. Gold

et al. 2008). Further study of testosterone treatment in men with MS is warranted.

Conclusion

Currently, MS treatments have a primary focus on reducing inflammation. Given that MS is both an inflammatory and neurodegenerative disease, there is a need for neuroprotective treatments if one aims to fully halt the disease (Meuth et al. 2010). Estrogens and androgens both have the potential to play neuroprotective roles in the treatment of MS. EAE studies with various estrogen and androgen treatments led to clinical disease protection, as well as protection from CNS inflammation, axonal loss, as well as demyelination. ERα ligand treatment was able to completely abrogate both early and late stages of disease. However, it is unlikely ERα ligand treatment could be used long term or in high doses since breast and ovarian cancers are mediated via ERα in humans (Bogush et al. 2009; Miyoshi et al. 2010). ERβ ligand and estriol treatments remain promising candidates for MS since they have the potential to be much safer due to either no, or relatively low, binding to ERα respectively (Kuiper 1997; Koehler et al. 2005; Younes & Honma 2011; Kuiper & Gustafsson 1997). In the EAE model, ERβ ligand was able to ameliorate late signs of clinical disease while providing neuroprotection even in the presence of CNS inflammation (Crawford et al. 2010; Tiwari-Woodruff et al. 2007). Recent evidence suggests that ERβ ligand may not only prevent demyelination, but also promote remyelination (Crawford et al. 2010). However, since ERβ ligand is does not prevent CNS inflammation, it would theoretically need to be given in combination with an anti-inflammatory agent in MS (Du, Sandoval, Trinh & Voskuhl 2010a). Androgen treatment remains a candidate for male MS patients. While some anti-inflammatory effects of testosterone have been shown in EAE, direct neuroprotective effects have not been addressed. It remains unclear whether the protective effect of testosterone might be due to its conversion to estrogen in the CNS.

Despite evidence in the preclinical MS model regarding estrogen's and androgen's potential as therapeutic treatments, more research is needed; particularly at the clinical level to assess the potential of sex hormone treatments for MS patients. Two trials are currently under

way to test the efficacy estriol and estradiol for women with MS. The first trial is a double blind placebo controlled trial of oral estriol given in combination with a standard of care treatment, Copaxone. RRMS subjects will be treated for two years, and the primary outcome measure is a reduction in relapses (http://www.clinicaltrials.gov/ct2/show/NCT00451204). The second trial, POTPART'MUS is a double blind trial aimed to prevent postpartum relapses. Women are given high doses of progestin in combination with physiological doses of estradiol immediately after delivery with continuous administration for the first 3 months post-partum (Vukusic et al. 2009). The field of MS anticipates the results of these trials.

FIGURES, TABLES, and LEGENDS

	EAE	EAE	EAE	MS
	Peripheral Splenocytes/LNCs	**Dendritic Cells**	**CNS**	**PBMCs**
ERα Ligand	↑ IL-5 {Morales: 2006dx, TiwariWoodruff: 2007dk} ↓TNFα, IFNγ, IL-2, IL-4, IL-6, IL-10, MMP9 {Elloso: 2005ig, Gold: 2006kc, Morales: 2006dx}	N/A	N/A	N/A
ERβ Ligand	↓IL-17 {Du:2010en}	↓TNFα {Confavreux: 1998bh}	N/A	N/A
Estradiol	↑ CCL2, CCL4, IL-18, TFGβ-3, CTLA-4, CCR3, VCAM {Bodhankar: 2011eg, Liu:2003ug, Matejuk:2002ty, Subramanian: 2003vd} ↓TNFα, IL-2, IFNγ, IL-6, IL-17, CCL2, CCL5, CCR1, CCR5, NCAM {Bodhankar: 2011eg, Liu:2003ug, Matejuk:2002ty, Subramanian: 2003vd}	↓TNFα, IFNγ, and IL-12 {Liu:2003ug}	↑ TGFβ-2, TGFβ-3, IL-1β, IL-17, CCL2, CCL5, CXCL13 {Bodhankar:2011eg, Matejuk:2001tl, Matejuk:2002ty, Subramanian: 2003vd} ↓ TNFα, IL-1β, IL-2, IFNγ, IL-4, IL-6, IL-17, CCL2, CCL3, CCL5, CXCL2, CXCL13, LT-β, TGFβ-1, CCR1, CCR2, CCR3, CCR4, CCR5, CCR7, CXCR5 {Bodhankar: 2011eg, Matejuk: 2001tl, Matejuk: 2002ty, Subramanian: 2003vd}	N/A
Estriol	↑ IL-5, IL-10, Il-4 {Kim:1999ti, Palaszynski:2004uh, Papenfuss:2004ky} ↓TNFα, IFNγ, IL-2, IL-6, MMP-9 {Gold: 2006kc, Kim:1999ti, Palaszynski:2004uh, Papenfuss:2004ky}	↑ TGF-β, IL-10, IL-12p35 {Papenfuss:2004ky} ↓ IL-12p40, IL-23p19, IL-6 {Papenfuss: 2004ky}	N/A	↑IL-5, IL-10 {Sicotte:2007fs, Soldan:2003ul} ↓ TNFα, IFNγ, MMP-9 {Sicotte:2007fs, Soldan:2003ul}

Testosterone/DHT	↑IL-10 {Bebo:2001tr, Dalal:1997vo} ↓IFNγ, TNFα {Bebo: 2001tr, Matejuk: 2005td}	N/A	N/A	↑BDNF, PDGF-BB, TGFβ1 {Gold:2008fb} ↓IL-2 {Gold: 2008fb}

Table 1. Treatment with exogenous estrogens and androgens provide immune protection in EAE and MS in multiple tissues/cells. Select cytokines/chemokines and their respective receptors as well as adhesion molecules, growth factors and proteinases are subject to increase or decrease with treatment of ERα ligand, ERβ ligand, estradiol (E2), estriol (E3), testosterone (T), and 5αDHT in splenocytes, lymph nodes, dendritic cells and CNS in EAE as well as PBMCs in MS patients. CNS includes mononuclear cells isolated from the CNS. Arrows indicate whether the change was an increase or decrease with the sex hormone treatment. Note: Some of the names of the molecules have been changed from their original name to reflect current nomenclature.

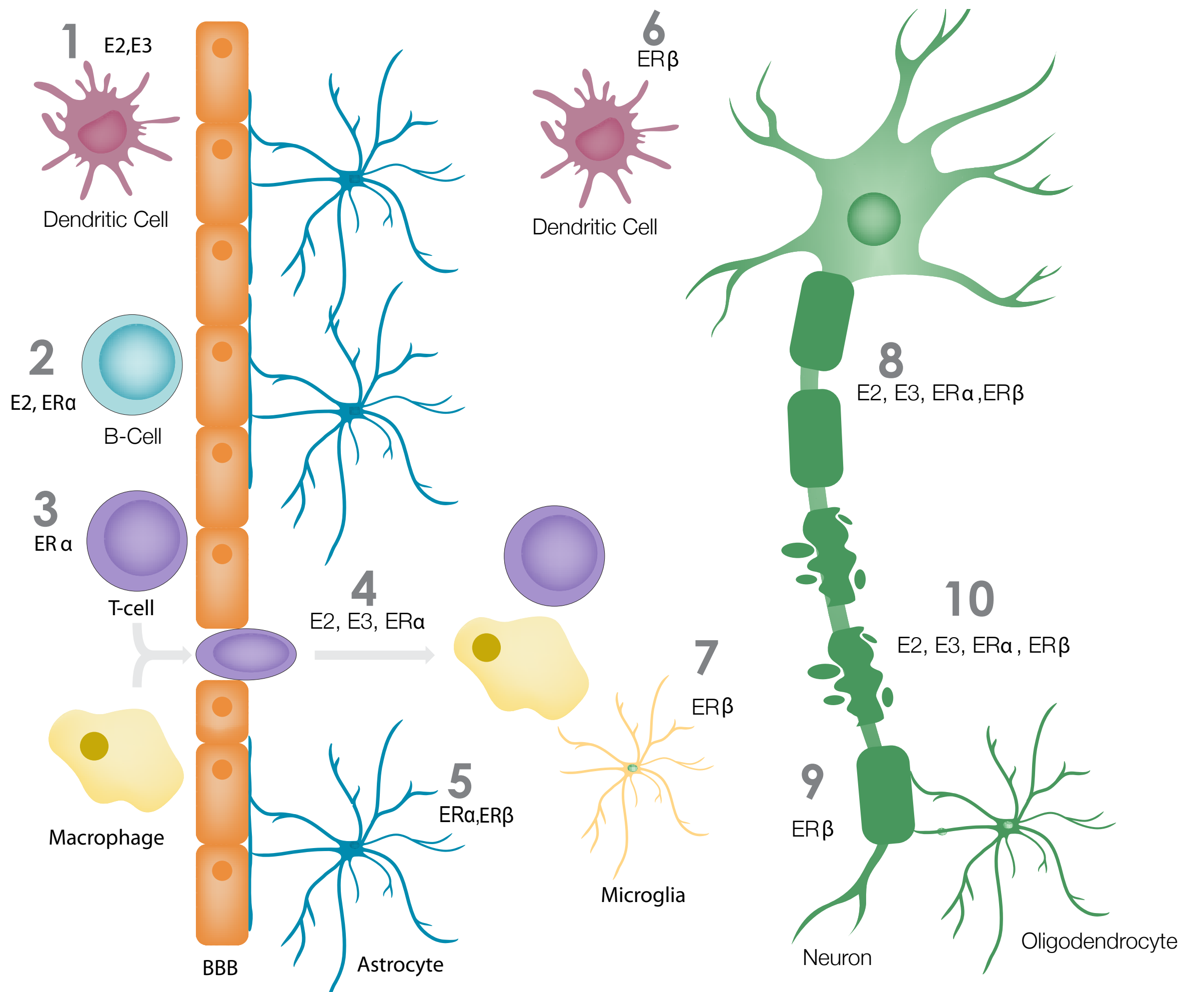

Figure 1. Simplified model of estrogen's actions for disease protection during EAE. 1) Estradiol (E2) and estriol (E3) act on dendritic cells (red) in the periphery to provide protection during EAE (H. Y. Liu et al. 2002; Papenfuss et al. 2011). 2) Estradiol and ERα ligand act on B-cells (light blue) to provide protection during EAE (Bodhankar et al. 2011). 3) ERα ligand acts on T-cells (purple) to prevent EAE (Lelu et al. 2011). 4) Estradiol, estriol, and ERα ligand prevent inflammation into the CNS during EAE (Bebo et al. 2001; Kim et al. 1999; Lelu et al. 2011; Morales 2006; Tiwari-Woodruff et al. 2007). 5) ERα and ERβ act on the astrocytes (dark blue) to promote disease protection (Saijo et al. 2011; Spence et al. 2011). 6) ERβ acts on dendritic cells (red) in the CNS to provide protection during EAE (Du, Sandoval, Trinh, Umeda, et al. 2010b). 7) ERβ acts on microglia to provide protection during EAE (Saijo et al. 2011) 8) Estradiol, estriol, ERα and ERβ ligand treatment during EAE prevent axonal loss (Bebo et al. 2001; Kim et al.

1999; Morales 2006; Tiwari-Woodruff et al. 2007). 9) ERβ ligand promotes remyelination of axons during EAE (Crawford et al. 2009). 10) Estradiol, estriol, ERα and ERβ ligand prevent demyelination during EAE (Bebo et al. 2001; Kim et al. 1999; Morales 2006; Tiwari-Woodruff et al. 2007).

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CHAPTER II

Estrogen Receptor Beta is Dispensable on Astrocytes and Neurons for Neuroprotection from Estrogen Receptor Beta Ligand Treatment during Experimental Autoimmune Encephalomyelitis

INTRODUCTION

Estrogens are neuroprotective in numerous animal disease models of the central nervous system (CNS) including Alzheimer's Disease (AD), Parkinson's Disease (PD), and Multiple Sclerosis (MS). This neuroprotection ranges from prevention of neuronal loss to proper maintenance of the mitochondria (Wise 2002; Spence & Voskuhl 2012; Brinton 2008; Lee & McEwen 2001). While therapeutic effects of estrogen treatment in experimental models of diseases have been shown, the cellular targets of estrogen treatment are not yet fully understood.

EAE is the most widely used mouse model of MS (Croxford et al. 2011; R. Gold 2006). Similar to MS, EAE consists of CNS inflammation, demyelination, and axonal loss (Trapp & Nave 2008). Estrogen treatment exerts well documented protective effects in EAE (Spence & Voskuhl 2012; S. M. Gold & Voskuhl 2009). Previous studies showed that treatment with estrogen receptor beta (ERβ) ligand ameliorated EAE, as well as prevented demyelination and axonal loss (Du et al. 2010; Crawford et al. 2010; Tiwari-Woodruff et al. 2007). ERβ ligand treatment was able to qualitatively affect CNS infiltration as well as promote remyelination (Crawford et al. 2009; Du et al. 2010).

Here, we determined whether ERβ ligand ameliorated EAE through direct actions on neurons and/or astrocytes. To do so, we employed a genetic loss-of-function strategy. We selectively deleted ERβ from neurons and astrocytes in two separate mouse lines using the well characterized *Cre-loxP* systems for neuronal conditional gene knockout (neuronal-CKO) or astrocyte conditional gene knockout (astrocyte-CKO) in mice to remove ERβ (neuronal-CKO-ERβ) or (astrocyte-CKO-ERβ) (Forss-Petter et al. 1990; Kwon et al. 2006; Herrmann et al. 2008; Spence et al. 2011). Our findings demonstrate that ERβ expression on neurons and astrocytes *in vivo* during EAE is not necessary for estrogen-mediated neuroprotection.

RESULTS

ERβ is Specifically Deleted from Astrocytes and Neurons in astrocyte-CKO-ERβ and neuronal-CKO-ERβ Mice Respectively. To target CKO-ERβ to neurons we used a rat neuronal specific enolase II promoter (rNSE II)-Cre line previously shown to target Cre activity selectively in neurons in the CNS (Forss-Petter et al. 1990; Kwon et al. 2006). To target CKO-ERβ to astrocytes we used a mouse glial fibrillary acid protein (mGFAP)-Cre line previously shown to target Cre activity selectively in astrocytes in the CNS (Herrmann et al. 2008). This mGFAP-Cre line is able to target 98% of all astrocytes with no targeting of other cells in the CNS, such as oligodendrocytes, microglia, and neurons. We then crossed these mouse lines separately with ERβ-loxP mice, in which exon 3 of ERβ gene is floxed (Dupont et al. 2000). To determine the efficacy of ERβ CKO from neurons and astrocytes, we performed immunohistochemistry in both WT, neuronal-CKO-ERβ, and astrocyte-CKO-ERβ mouse lines with EAE. Our results demonstrated that ERβ was absent in neurons in the neuronal-CKO-ERβ mouse line while it was present in neurons of both WT and astrocyte-CKO-ERβ mice (Fig. 1A). We also showed that ERβ was absent in astrocytes in the astrocyte-CKO-ERβ mouse line while it was present in astrocytes of both the WT and neuronal-CKO-ERβ mouse line (Fig. 1B).

ERβ Ligand Does Not Act Directly on Neurons or Astrocytes for Clinical Disease Protection. To determine if neurons or astrocytes were the target of ERβ ligand mediated protection during EAE, we treated our neuronal-CKO-ERβ and astrocyte-CKO-ERβ mice with ERβ ligand during the effector phase of adoptive EAE (Kim et al. 1999). WT mice with EAE treated with vehicle exhibited a level of clinical disease indistinguishable from neuronal-CKO-ERβ or astrocyte-CKO-ERβ mice with EAE treated with vehicle, demonstrating that the removal of neuronal and astrocyte ERβ did not affect the clinical course of disease (Fig. 1 C/D). WT mice with EAE treated with ERβ ligand showed significantly less clinical disease than WT mice with EAE treated with vehicle, confirming that ERβ ligand treatment is able to ameliorate clinical

disease (Du et al. 2010; Morales 2006; Tiwari-Woodruff et al. 2007). Notably, ERβ ligand treatment was still able to ameliorate clinical disease in the neuronal-CKO-ERβ and astrocyte-CKO-ERβ group, demonstrating that neurons and astrocytes are not the target of ERβ ligand mediated clinical disease protection (Fig. 1C/D).

ERβ Ligand Treatment Does Not Prevent T-cell and Macrophage Inflammation in the CNS. CNS inflammation, comprised principally of T-cells and monocytes, are a hallmark of EAE (Tiwari-Woodruff et al. 2007; Voskuhl et al. 2009). Thus, we used immunohistochemistry to quantify the levels of T-cells and monocytes in ERβ ligand treated EAE neuronal-CKO-ERβ and astrocyte-CKO-ERβ mouse lines. Anti-CD3 antibody was used to detect T-cells while anti-Iba-1 antibody was used to detect Iba-1 ramified microglia and Iba-1 globoid macrophages as previously described (Voskuhl et al. 2009). T-cells were increased in all mice with EAE. ERβ ligand treatment was unable to reduce T-cell infiltration in either WT, neuronal-CKO-ERβ, and astrocyte-CKO-ERβ mouse lines (Fig. 2A, Fig. 3 A/C). In addition, Iba-1 globoid macrophages were increased in all mice with EAE. ERβ ligand treatment was unable to reduce Iba-1 globoid macrophage infiltration in either WT, neuronal-CKO-ERβ, and astrocyte-CKO-ERβ mouse lines (Fig. 2 C, Fig. 3 B/D). Also, when Iba-1 ramified microglia were examined, there were no differences between groups (Fig. 2B, Fig. 3 B/D). These results confirm previous results which demonstrate that ERβ ligand treatment is not able to quantitatively affect immune cell infiltration into the CNS (Tiwari-Woodruff et al. 2007).

ERβ Ligand Does Not Act on Neurons or Astrocytes to Prevent Demyelination, Astrogliosis and Axonal Loss in the CNS. We next examined classical neuropathology of EAE by quantifying differences in myelin, gliosis, and axons in the spinal cord (Du et al. 2010; Morales 2006; Tiwari-Woodruff et al. 2007; Voskuhl et al. 2009). To quantify myelin, we counted the number of axons using anti-neurofilament 200 (NF200) that were fully encompassed by a ring of myelin basic protein (MBP). ERβ ligand treatment of WT mice with EAE ameliorated

demyelination compared to WT mice with EAE treated with vehicle, confirming the protective effect of ERβ ligand on myelin loss (Du et al. 2010; Tiwari-Woodruff et al. 2007) (Fig. 4, Fig. 5A and D). Interestingly, ERβ ligand treatment was able to prevent demyelination in both neuronal-CKO-ERβ and astrocyte-CKO-ERβ mice, suggesting that ERβ ligand acts upon a cell other than neurons or astrocytes to protect against myelin loss (Fig. 4, Fig. 5A and D). Axonal loss is known to correlate with clinical disease scores (Wujek et al. 2002). Thus, we counted the number of axons using NF200. ERβ ligand treatment of WT mice with EAE prevented axonal loss compared to WT mice with EAE treated with vehicle, confirming the protective effect of ERβ ligand on axonal loss (Du et al. 2010; Tiwari-Woodruff et al. 2007)(Fig. 4B, Fig. 5B and E). Interestingly, ERβ ligand treatment was able to prevent axonal loss in neuronal-CKO-ERβ and astrocyte-CKO-ERβ mice, suggesting that ERβ ligand acts upon a cell other than neurons or astrocytes to protect against axonal loss (Fig. 4B, Fig. 5B and E). To quantify reactive astrogliosis, glial acidic fibrillary protein (GFAP) expression was assessed. ERβ ligand treatment had no effect on astrogliosis in any of the groups with EAE (Fig. 4C and Fig. 5F), suggesting that ERβ ligand is not able to decrease reactive astrogliosis in the CNS.

DISCUSSION

We show that neuroprotection in EAE mediated via ERβ signaling does not require ERβ on neurons or astrocytes. These findings have important implications for understanding the different possible means by which estrogens can ameliorate EAE and MS, and for strategies to exploit the potential of selective ERβ ligands in treatment strategies for MS and other neuroinflammatory conditions.

Previous studies from various laboratories have shown that estrogens exert neuroprotective effects in EAE that consist of a decrease in clinical disease, a reduction of CNS inflammation, and a decrease in axonal loss (Elloso 2005; Polanczyk et al. 2010; Spence & Voskuhl 2012). Our findings here extend these observations by showing that estrogens can exert neuroprotection in EAE when signaling through ERβ. Consistent with previous studies, we found that ERβ-ligand decreased clinical disease, demyelination, and axonal loss in EAE (Tiwari-Woodruff et al. 2007; Crawford et al. 2009; Du et al. 2010). Interestingly, selective deletion of ERβ from neurons and astrocytes had no effect on the neuroprotective effects of ERβ ligand. The neuroprotective effects of ERβ ligand must be mediated by some other cell type, perhaps by acting on microglia, dendritic cells, or oligodendrocytes (Saijo et al. 2011; Du et al. 2010; Crawford et al. 2010).

The neuroprotective effects of estrogens, as well as their ligands that bind specifically to ERβ, are under consideration and investigation for potential therapeutic application in various clinical conditions, including MS. Still, while neurons and astrocytes do not appear to be the target of ERβ ligand's neuroprotective effects, it does not remove the possibility that these same cells could be the target of ERα ligand's neuroprotection during EAE. Thus, in the following chapters we will explore this possibility.

MATERIALS AND METHODS

Animals

All mice were on a C57BL/6 background achieved by at least 10 generations of back crossing. Neuronal-CKO-ERβ were generated by crossing mice of rNSE II-Cre line (Forss-Petter et al. 1990; Kwon et al. 2006) with mice carrying an ERβ gene in which exon 3 was flanked by loxP sites (ERβ$^{flox/flox}$) were the generous gift of Professor Pierre Chambon (Strasbourg) (Dupont et al. 2000). Astrocyte-ERβ-CKO were generated by crossing mice of mGFAP-Cre line 73.12 (Herrmann et al. 2008) with mice carrying an ERβ gene in which exon 3 was flanked by loxP sites (ERβ$^{flox/flox}$) were the generous gift of Professor Pierre Chambon (Strasbourg) (Dupont et al. 2000). Animals were maintained under standard conditions in a 12-hour 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.

Adoptive EAE and Hormone Manipulations

C57BL/6 donor animals were immunized subcutaneously with Myelin Oligodendrocyte Glycoprotein (MOG), amino acids 35-55 (200 μg/animal, American Peptides) emulsified in Complete Freund's Adjuvant (CFA), supplemented with *Mycobacterium Tuberculosis H37Ra* (200 μg/animal, Difco Laboratories), over four sites drained by inguinal and auxiliary lymph nodes in a total volume of 0.1 mL/mouse. Immunized mice had lymph node cells (LNCs) cultured in 24-well plates at a concentration of $3x10^6$ 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-hours. On the third day of culture, LNCs were washed with 1X PBS and each recipient mouse received $3x10^7$ cells in 0.3ml ice-cold PBS by intraperitoneal injection. Recipient female C57Bl/6 WT and astrocyte-CKO mice had been gonadectomized at

4 weeks of age, and had EAE induced by adoptive transfer at 8 weeks of age. Recipient mice were treated every other day with the ERβ ligand (DPN) (Tocris) at the dose of 8 mg/kg/day or vehicle diluted with 10% molecular-grade ethanol (EM Sciences) and 90% Miglylol 812N liquid oil (Sasol North America) beginning seven days before adoptive transfer. These doses of PPT and DPN have been previously established (Tiwari-Woodruff et al. 2007). Animals were monitored daily for EAE signs based on a standard EAE 0-5 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 1X PBS for 20-30 minutes, followed by 10% formalin for 10-15 minutes. Spinal cords were dissected and submerged in 10% formalin overnight at 4°C, followed by 30% sucrose for 24 hours. Spinal cords were cut in thirds and embedded in optimal cutting temperature compound (Tissue Tek) and frozen at -80°C. 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 0.1M PBS with 1% sodium azide in 4°C until immunohistochemistry.

Immunohistochemistry

Prior to histological staining, 40 μm thick free-floating sections were thoroughly washed with 0.1M PBS to remove residual sodium azide. For tissues to be treated with diaminobenzidine (DAB), sections were permeabilized with 0.5% Triton X-100 in 0.1M TBS and 10% normal goat serum (NGS) for 60 minutes at RT. 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), anti-glial fibrillary acidic protein-GFAP at 1:40,000 (Dako), anti-ERβ at 1:5,000 (Upstate), and anti-myelin basic protein-MBP at 1:750 (Sigma). Tissues were then washed three times for 10 minutes in 0.1M TBS. Tissues were

labeled with secondary antibodies conjugated to Cy5 or Cy3 (Vector Labs and Chemicon) for 1 hour for NF-200, MBP, Iba-1, CD3, 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 semi-dry, and cover slipped in fluoromount G (Fisher Scientific). DAB sections were dried over night and then dehydrated in 70%, 95% and 100% ethonal, followed by 5 minutes of Ctitrasolve and coverslipped with Permount (Fisher). IgG-control experiments were performed for all primary antibodies, and only non-immunoreactive tissues under these conditions were analyzed. IHC for ERβ was followed directly as previously described (Giraud et al. 2010).

Quantification

To quantify immunohistochemical staining results, three dorsal column spinal cord cross-sections at the T1-T5 level from each mouse were captured under microscope at 10X or 40X 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 downloaded from the NIH website (http://rsb.info.nih.gov/ij). Three sections from each animal were then quantified to calculate the mean per animal. Immunohistochemical experiments were combined from three separate clinical trials. In order 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. Myelination was calculated by counting the number of $NF200^+$ cells fully encircled by myelin basic protein (MBP). Axonal densities were calculated by counting the number of $NF200^+$ cells in a 40X 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 40x under a light microscope. GFAP were calculated as percent intensity or area respectively from the dorsal column.

Microscopy

Stained sections were examined and photographed using a confocal microscope (Leica TCS-SP, Mannheim, Germany) or a fluorescence microscope (BX51WI; Olympus, Tokyo, Japan) 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, San Jose, CA) and Microsoft PowerPoint. DAB sections were examined at the light level at 40x (Nikon Alphaphot-2 YS2).

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).

FIGURES and LEGENDS

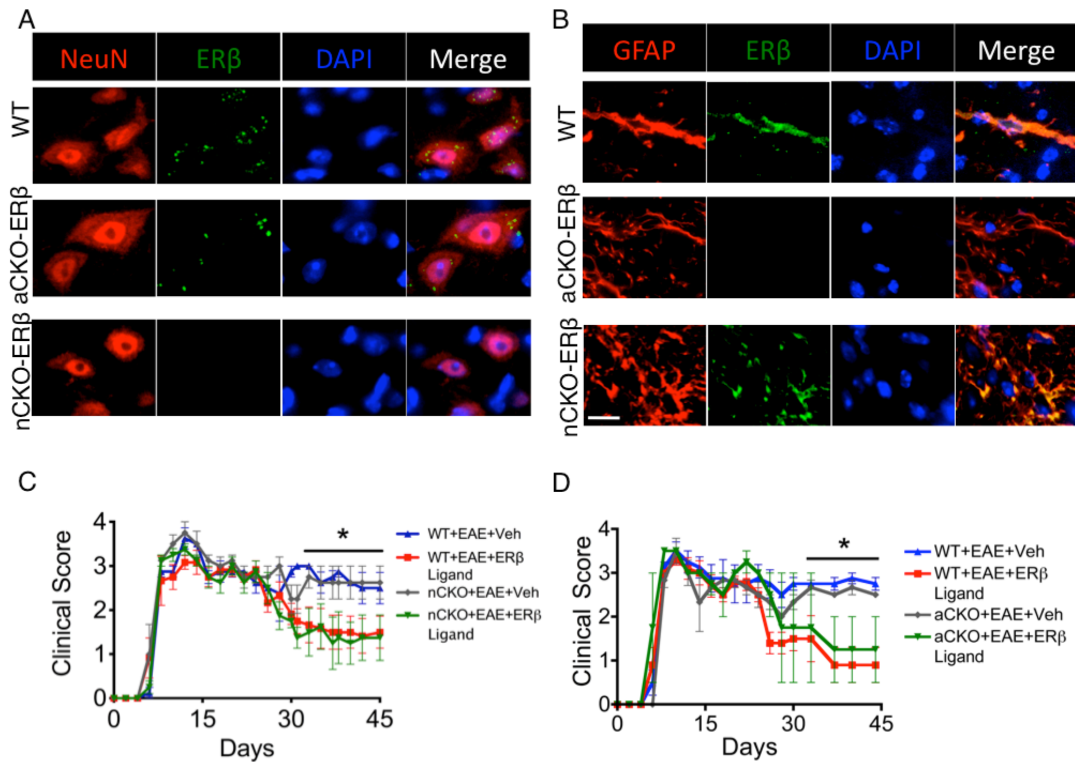

Figure 1. Verification of gene deletion specificity in neuronal-CKO-ERβ (nCKO-ERβ) and astrocyte-CKO-ERβ (aCKO-ERβ) mouse models and EAE clinical disease severity scores showing that protective effects of ERβ are not dependent on ERβ expression of neurons and astrocytes. (A) Immunohistochemistry shows ERβ colocalized with NeuN and DAPI in WT and aCKO-ERβ, but not nCKO-ERβ, mice with EAE (Scale bar, 12μm.). (B) Immunohistochemistry shows ERβ colocalized with GFAP and DAPI in WT and nCKO-ERβ, but not aCKO-ERβ, mice with EAE. (C) WT and nCKO-ERβ mice treated with ERβ ligand had significantly better clinical scores compared with WT or nCKO-ERβ mice with EAE treated with vehicle. (D) WT and aCKO-ERβ mice treated with ERβ ligand had significantly better clinical scores compared to WT or aCKO-ERβ mice with EAE treated with vehicle. n=10 per group.*$P<0.05$ (repeated-measures ANOVA with post hoc Bonferroni pairwise analysis).

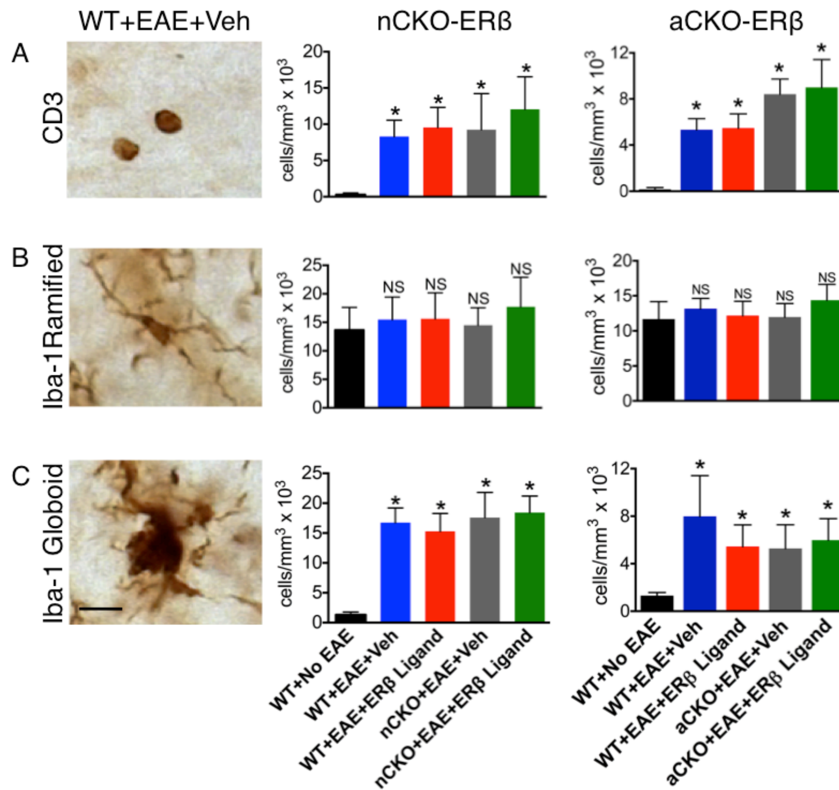

Figure 2. ERβ expression is not required in neurons or astrocytes to reduce the numbers of CD3 T-cells and Iba-1 globoid macrophages in EAE spinal cord. (A) CD3 T-cells were not reduced in WT or neuronal-CKO-ERβ (nCKO-ERβ) mice with EAE treated with ERβ ligand. Treatment with ERβ ligand was unable to reduce CD3 T-cells in WT or astrocyte-CKO-ERβ (aCKO-ERβ) with EAE. (B) Iba-1 ramified microglia exhibited no significant difference in number across all experimental groups. (C) Iba-1 globoid macrophages were not significantly reduced in WT or nCKO-ERβ mice treated with EAE treated with ERβ ligand. Iba-1 globoid macrophages were not significantly reduced in WT or aCKO-ERβ mice treated with EAE treated with ERβ ligand.(Scale bar, 12µm.) n=6 per group. * =$P<0.05$ vs WT+No EAE; NS=not significant vs. WT +No EAE, (ANOVA with post hoc Bonderroni pairwise analysis).

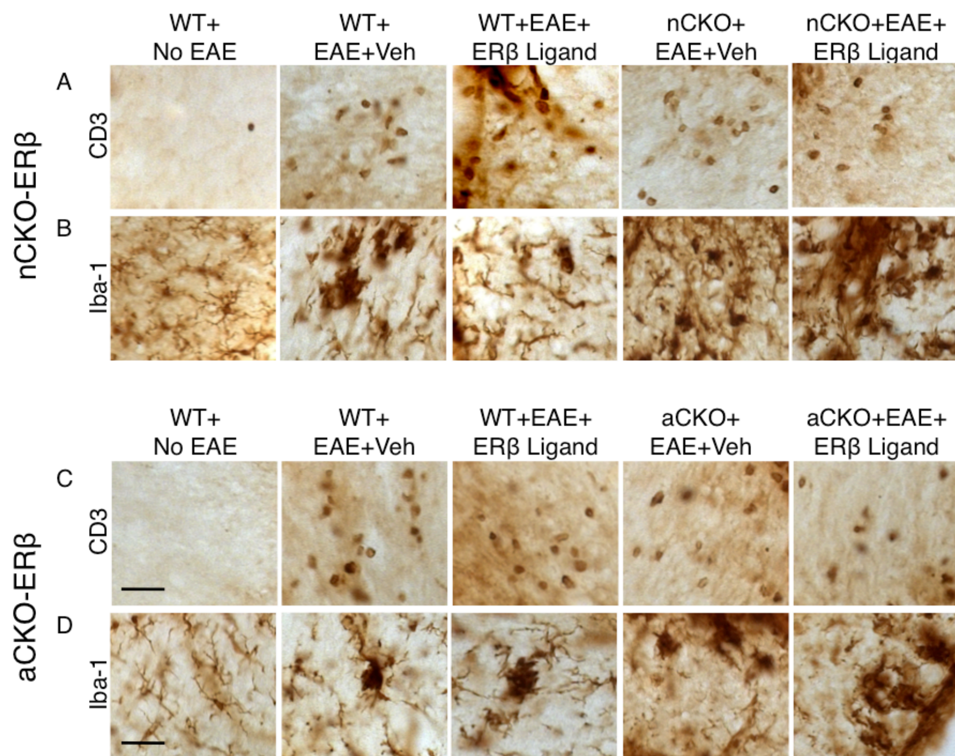

Figure 3. ERβ is not required in neurons or astrocytes to reduce the numbers of CD3 T-cells and Iba-1 globoid macrophages in EAE spinal cord. (A and B) CD3 T-cells and Iba-1 globoid macrophages are increased in WT and neuronal-CKO-ERβ (nCKO-ERβ) mice with EAE compared with WT mice without EAE. Treatment with ERβ ligand does not ameliorate this increase in WT mice and nCKO-ERβ mice with EAE. (C and D) CD3 T-cells and Iba-1 globoid macrophages are increased in WT and astrocyte-CKO-ERβ (aCKO-ERβ) mice with EAE compared to WT mice without EAE. Treatment with ERβ ligand was not able to ameliorate this increase in both WT and aCKO-ERβ mice. [Scale bar, 21μm (CD3) and 17μm (Iba-1)].

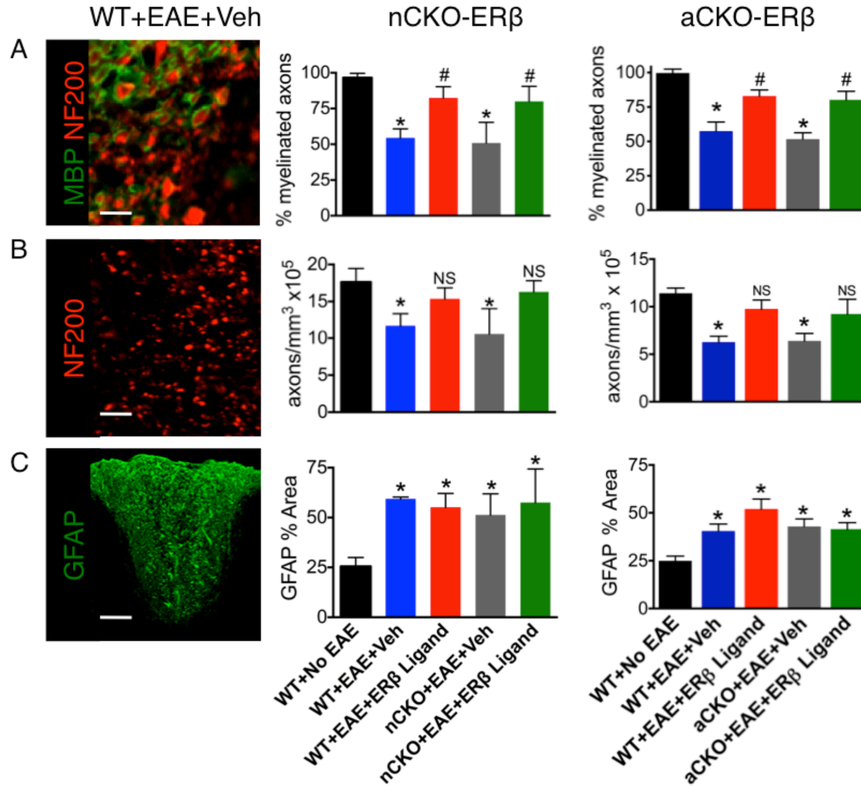

Figure 4. ERβ, is not required in neurons or astrocytes to protect against demyelination, axonal loss, and reactive astrogliosis. (A) NF200 axons fully encased by MBP were significantly reduced in WT mice with EAE, and treatment with ERβ ligand prevented demyelination in WT and neuronal-CKO-ERβ (aCKO-ERβ) mice. Treatment with ERβ ligand was also able to prevent demyelination in WT and astrocyte-CKO-ERβ (aCKO-ERβ) mice. (Scale bar, 15μm.) (B) Numbers of NF200 axons were significantly reduced in WT mice with EAE, and treatment with ERβ ligand prevented axonal loss in WT and nCKO-ERβ mice. Treatment with ERβ ligand was also able to prevent axonal loss in WT and aCKO-ERβ mice. (Scale bar, 40μm.) (C) GFAP stained area was significantly increased in WT mice with EAE, and treatment with ERβ ligand did not prevent this increase in WT and nCKO-ERβ mice with EAE. Treatment with ERβ ligand was unable to prevent reactive gliosis in WT or aCKO-ERβ mice with EAE. (Scale bar, 122μm.) n=6 per group. *=P<0.05 vs WT+No EAE; #=P<0.05 vs WT+No EAE and EAE+Veh; NS=not significant vs WT+No EAE, (ANOVA with post hoc Bonferroni pairwaise analysis).

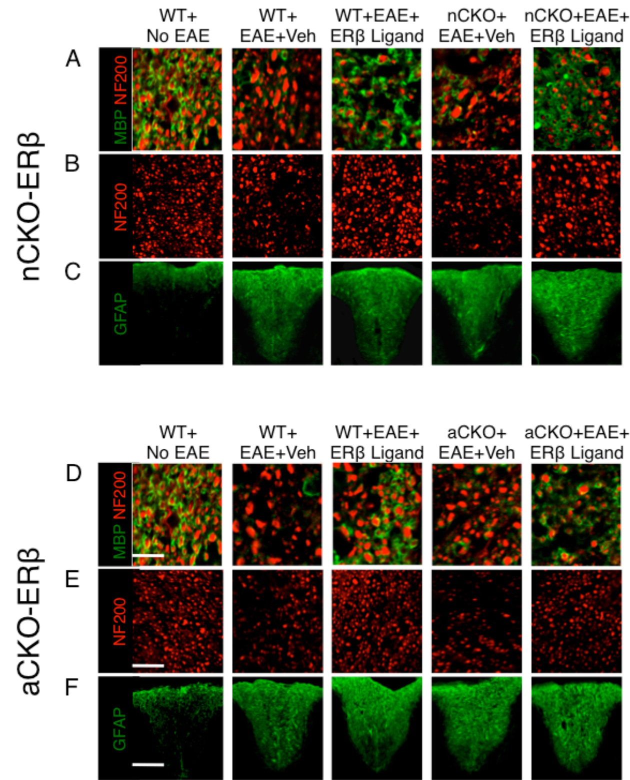

Fig. 5. ERβ is not required on neurons or astrocytes to protect against demyelination, axonal loss, and gliosis in EAE spinal cord. (A and D) NF200 axons fully encompassed by MBP exhibit a decrease in WT mice with EAE. Treatment with ERβ ligand prevented demyelination in WT and neuronal-CKO-ERβ (nCKO-ERβ) mice with EAE. Treatment with ERβ ligand prevented demyelination in both WT and astrocyte-CKO-ERβ (aCKO-ERβ) mice with EAE. (Scale bar, 22μm). (B and E) NF200 axons exhibit patchy reduction in numbers in WT mice with EAE. Treatment with ERβ ligand prevented axonal loss in WT and nCKO-ERβ mice with EAE. Treatment with ERβ ligand prevented axonal loss in both WT and aCKO-ERβ mice with EAE. (Scale bar, 50μm). (C and F) GFAP stained area increased in WT mice with EAE. Treatment with ERβ ligand was unable to decrease reactive gliosis in WT and nCKO-ERβ mice with EAE. Treatment with ERβ ligand was unable to decrease reactive gliosis in WT and aCKO-ERβ mice with EAE. (Scale bar, 130μm).

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CHAPTER III

Estrogen Receptor Alpha is Necessary on Astrocytes but not Neurons for Neuroprotection from Estrogen Receptor Alpha Treatment during Experimental Autoimmune Encephalomyelitis

INTRODUCTION

The female sex hormone, estrogen, is neuroprotective in many clinical and experimental CNS disorders, including autoimmune conditions such as multiple sclerosis (MS), neurodegenerative conditions such as Alzheimer's and Parkinson's Diseases, and traumatic injury, and stroke (Brinton 2008; Lee & McEwen 2001; Wise 2002; Kim et al. 1999). Estrogen treatment has been shown to ameliorate clinical disease and decrease neuropathology in these disease models (Brinton 2008; Lee & McEwen 2001; Wise 2002; Kim et al. 1999). Pharmacological studies have suggested roles for different estrogen receptors, but the cell type(s) that mediate neuroprotective effects of estrogen are not known for any experimental or clinical condition. Identifying cells that bear specific estrogen receptor subtypes and are essential for specific estrogen-mediated effects is fundamental to elucidating and therapeutically exploiting the mechanisms that underlie estrogen-mediated neuroprotection. Towards this end, we used a genetic loss-of-function strategy. We selectively deleted estrogen receptor alpha (ERα) from two different CNS cell types, neurons and astrocytes, and then determined the effects of these conditional gene deletions on the ability of ERα-ligand treatment to ameliorate disease severity of experimental autoimmune encephalomyelitis (EAE) in mice.

EAE is the most widely used mouse model of MS, and like MS, is a CNS autoimmune disease characterized by demyelination and axonal degeneration (Trapp & Nave 2008). Estrogen exerts a beneficial effect on the clinical course and neuropathology of EAE that is mediated at least in part by ERα, as shown by studies using ERα selective ligands or global gene deletion (Morales 2006; Polanczyk et al. 2010). ERα ligand treatment in EAE significantly ameliorates clinical symptoms and reduces both inflammation and axonal loss in spinal cord (Tiwari-Woodruff et al. 2007). The cell type(s) mediating the effects of ERα in EAE are not known because ERα is expressed by various immune cells as well as CNS neurons and glia (Garidou et al. 2004; Pérez et al. 2003; Garcia-Ovejero et al. 2002; Mitra 2003). Bone marrow chimera studies revealed that ERα expression in the peripheral immune system is not required for estradiol mediated protection during EAE (Garidou et al. 2004), suggesting that ERα

expression on a CNS cell type is critical for ERα mediated neuroprotection. Although neurons are an obvious potential CNS target of estradiol and ERα ligand mediated protection, astrocytes, a CNS glia with many complex functions in health and disease (Barres 2008; Freeman 2010), represent an alternative candidate cell that has been implicated in regulation of CNS inflammation and neuroprotection *in vivo* in various models of CNS diseases, including EAE (Voskuhl et al. 2009; Sofroniew 2009).

In this study we tested the hypothesis that CNS neuroprotective effects of estrogen mediated through ERα *in vivo* are effectuated via expression of ERα in either neurons or astrocytes. We selectively deleted estrogen receptor alpha (ERα) in either neurons or astrocytes using well-characterized Cre-loxP systems for conditional gene knockout (CKO) in mice. We then studied the effects of adoptive EAE (Kim et al. 1999) in neuronal-ERα-CKO (nCKO-ERα), astrocyte-ERα-CKO (aCKo-ERα) and wild type (WT) mice that were gonadectomized and treated with ERα ligand or vehicle. Gonadectomized mice were used to avoid the potential confound of various circulating sex hormones. Our findings show that signaling through ERα in astrocytes, but not in neurons, is essential for the neuroprotective effects of systemic treatment with ERα ligand on clinical function, CNS inflammation and axonal loss during EAE.

RESULTS

ERα is Specifically Deleted from either Neurons or Astrocytes in the Respective Neuronal-ERα-CKO or Astrocyte-ERα-CKO Models. To target ERα-CKO to neurons, we used a rat neuronal specific enolase (rNSE)-Cre line previously shown at the single cell level to reliably target Cre activity selectively to essentially all neuronal cells throughout the brain and spinal cord, with no targeting of astrocytes, microglia or oligodendrocytes (Kwon et al. 2006; Forss-Petter et al. 1990). To target ERα-CKO to astrocytes, we used a mouse glial fibrillary acid protein (mGFAP)-Cre line previously shown at the single cell level to reliably target Cre activity selectively to essentially all astrocytes throughout the brain and spinal cord (Herrmann et al. 2008). Previous evaluation of reporter protein expression of over 2000 cells at the single cell level, quantitatively demonstrated that this mGFAP-Cre line reliably targets Cre activity to over 98% of reactive astrocytes, with no targeting of neurons, microglia, or oligodendrocytes in traumatically injured spinal cord. In addition, no cortical or brainstem neurons that project into the spinal cord are targeted by Cre-activity as shown by retrograde tract-tracing (Herrmann et al. 2008). These neuronal or astrocyte Cre mice were crossed with previously well characterized ERα-loxP mice (Dupont et al. 2000).

To determine the efficacy and selectivity of ERα deletion in either neurons or astrocytes we assessed levels of immunohistochemically detectable ERα using a well-characterized antibody (Giraud et al. 2010). WT mice with EAE exhibited readily detectable immunoreactive ERα in CNS neurons (Fig. 1A) and astrocytes (Fig. 1B). Neuronal-ERα-CKO mice with EAE did not express ERα at detectable levels in neurons (Fig. 1A) but did express ERα in astrocytes at levels that were indistinguishable from those in WT mice (Fig. 1B). Astrocyte-ERα-CKO mice with EAE did not express ERα at detectable levels in astrocytes (Fig. 1B) but did express ERα in neurons at levels that were indistinguishable from those in WT mice (Fig. 1A). In addition, ERα mRNA and protein, as detected by RT-PCR (Fig. 2A/C) or immunoblot (Fig. 2B/C), respectively, were essentially absent in astrocyte cultures from astrocyte-ERα-CKO mice, while

present in astrocytes from WT mice. Together, these findings demonstrate both the efficacy and specificity of our models for conditional ERα deletion from either neurons or astrocytes. Untreated neuronal-ERα-CKO mice and astrocyte-ERα-CKO mice were behaviorally and histologically indistinguishable from WT mice.

ERα Expression is Necessary in Astrocytes, but not Neurons, for Clinical Disease Protection. We first determined the clinical effect of ERα ligand treatment starting 7 days prior to adoptive EAE induction (Kim et al. 1999) in separate experiments that compared neuronal-ERα-CKO mice or astrocyte-ERα-CKO with their respective controls (Fig. 1C/D). Neuronal-ERα-CKO mice (Fig. 1C) and astrocyte-ERα-CKO mice (Fig. 1D) treated with vehicle exhibited EAE courses that were indistinguishable in clinical severity from those of vehicle-treated WT mice, whereas ERα ligand treated WT mice exhibited significantly less severe clinical disease (Fig. 1C/D), demonstrating a protective effect of ERα ligand when administered in the effector phase of adoptive EAE. Neuronal-ERα-CKO mice treated with ERα ligand also exhibited significant amelioration of clinical disease that was of a level comparable to that seen in WT EAE mice treated with ERα ligand (Fig. 1C). In striking contrast, astrocyte-ERα-CKO mice treated with ERα ligand exhibited severe clinical disease that was indistinguishable in severity from that of vehicle treated WT EAE mice (Fig. 1D). Together these observations demonstrate that ERα expression in astrocytes, but not in neurons, is required for the protective effects of ERα ligand on EAE clinical scores.

ERα Expression is Necessary in Astrocytes, but not Neurons, to Prevent Macrophage and T-cell Inflammation in the CNS. Following final assessments of clinical scores on day 25 after initiation of EAE, all mice were either fixed by cardiac perfusion for histopathological evaluation or processed for flow cytometry. CNS inflammation during EAE includes both T-cells and cells of the monocyte lineage (Tiwari-Woodruff et al. 2007; Voskuhl et

al. 2009). We first used immunohistochemical identification and a stereological procedure (StereoInvestigator ®) to quantify these cell types in the spinal cord dorsal column (Fig. 3).

To identify cells of the monocyte lineage we used immunohistochemistry for Iba-1 and divided positive cells into two phenotypes, those with a globoid shape associated with monocytes and phagocytic macrophages (Fig. 3A), and those with a ramified shape associated with CNS resident microglia (Fig. 3B) as previously described (Voskuhl et al. 2009). Vehicle treated WT, neuron-ERα-CKO, and astrocyte-ERα-CKO mice with EAE all exhibited significantly greater numbers of globoid Iba-1 stained cells compared to WT mice without EAE (Fig. 3A), in a manner consistent with macrophage infiltration in the CNS of EAE mice. ERα ligand treatment significantly reduced the numbers of globoid Iba-1 cells in WT and neuron-ERα-CKO mice with EAE to levels indistinguishable from WT mice without EAE, but had no effect in astrocyte-ERα-CKO mice with EAE, which had numbers indistinguishable from vehicle treated EAE mice (Fig. 3A, Fig. 4A/C). There were no differences in the number of ramified Iba-1 microglia among the four experimental groups (Fig. 3B).

To identify T-cells, we used immunohistochemistry for CD3 (Fig. 3C, Fig. 4B/D). Vehicle treated WT, neuron-ERα-CKO, and astrocyte-ERα-CKO mice with EAE all exhibited significantly greater numbers of CD3 stained T-cells in the spinal cord dorsal columns compared to WT mice without EAE (Fig. 3C). ERα ligand treatment significantly reduced the numbers of CD3 T-cells, in WT and neuron-ERα-CKO mice with EAE to levels indistinguishable from WT mice without EAE, but had no effect in astrocyte-ERα-CKO mice with EAE, which had numbers of CD3 T-cells indistinguishable from vehicle treated EAE mice (Fig. 3C, Fig. 4B/D).

To compliment and extend the immunohistochemical data, we used flow cytometry, focusing on the astrocyte-ERα-CKO mice in which the effects of ERα ligand treatment examined thus far had been significantly abrogated. ERα ligand treatment significantly reduced the numbers of $CD11b^{hi}/CD45^{hi}$ macrophages and of $CD45^{hi}/CD3^{hi}$ T-cells in the CNS of WT mice with EAE as compared to vehicle treated WT mice with EAE, but had no effect on the numbers of these cells in astrocyte-ERα-CKO mice with EAE (Fig. 5). As with immunohistochemistry,

there were no differences in the number of microglia ($CD11b^{hi}/CD45^{int}$) among the four experimental groups (Fig. 5).

Together, these immunohistochemical and flow cytometry data demonstrate that ERα expression in astrocytes, but not in neurons, is required for the ability of ERα ligand treatment to significantly ameliorate macrophage and T-cell infiltration into CNS parenchyma, the hallmarks of CNS inflammation in EAE.

ERα Expression is Necessary in Astrocytes, but not Neurons, to Attenuate Axonal loss and Gliosis. We next evaluated EAE neuropathology, which is characterized by patchy demyelination and axonal loss in spinal cord white matter (Tiwari-Woodruff et al. 2007; Voskuhl et al. 2009; Wujek et al. 2002). Axonal loss correlates with clinical disease severity (Wujek et al. 2002). To assess demyelination we quantified the area occupied by immunofluorescence staining of myelin basic protein (MBP) in the spinal cord dorsal column. Compared with WT mice without EAE, mice from all EAE experimental groups exhibited many patchy areas of severe loss of MBP staining (Fig. 7A/D) and average levels of MBP staining were significantly lower (Fig. 6A), but there were no significant differences among any of the EAE treatment groups (Fig. 6A), suggesting that ERα ligand treatment did not act to protect against myelin loss. To determine axon numbers in the spinal cord dorsal column we used immunofluorescence staining for neurofilament 200 (NF200) and semi-automated counting software (Image J) (Voskuhl et al. 2009). Vehicle treated WT, neuron-ERα-CKO, and astrocyte-ERα-CKO mice with EAE all exhibited patchy areas of axon loss (Fig. 7B/E) and average numbers of axons were significantly lower compared to WT mice without EAE (Fig. 6B). EAE WT and neuron-ERα-CKO mice treated with ERα ligand exhibited axon numbers not significantly different from WT mice without EAE (Fig. 6B). In contrast, astrocyte-ERα-CKO mice with EAE treated with ERα ligand had axon numbers not significantly different from vehicle treated EAE mice (Fig. 6B), demonstrating that ERα expression in astrocytes, but not in neurons, is required for the protective effects of ERα ligand treatment on axon number in EAE.

Astrogliosis is prominent in areas of tissue pathology in EAE (Voskuhl et al. 2009). We quantified the area occupied by immunofluorescence staining for GFAP, the canonical marker for reactive astrocytes (Sofroniew 2009). ERα ligand treatment significantly reduced the areas of GFAP staining in dorsal column white matter of both WT and neuron-ERα-CKO mice with EAE, to levels indistinguishable from WT mice without EAE, but had no effect on GFAP staining area in astrocyte-ERα-CKO mice with EAE (Fig. 6C, Fig. 7C/F), demonstrating that ERα expression in astrocytes, but not in neurons, is required for the reduction in reactive astrogliosis mediated by ERα ligand treatment.

DISCUSSION

Together these data demonstrate that neuroprotective effects of ERα ligand treatment in EAE, including improved clinical function, reduced white matter inflammatory cell infiltrate, and axonal sparing, are dependent on signaling through ERα in astrocytes, but not neurons. The findings do not exclude additional effects of estrogens or related steroids that might be mediated by ERβ, or non-classical effects, on astrocytes or other CNS cell types including neurons (Tiwari-Woodruff et al. 2007; Dupont et al. 2000; Giraud et al. 2010). Sexual dimorphism is increasingly recognized in CNS molecular mechanisms (Jazin & Cahill 2010), and circulating levels of endogenous or administered estrogens influence disease severity in a wide variety of CNS disorders including autoimmune inflammation (Kim et al. 1999), traumatic injury (Chaovipoch et al. 2006; Dubal et al. 2001), stroke (Wise 2002) and neurodegenerative disease (Brinton 2008; Wise 2002; Sherwin 2007), all of which have postulated inflammatory involvement. Our findings show that astrocytes are the principal cells required for mediating the neuroprotective effects ERα signaling in an autoimmune CNS inflammatory condition.

Astrocytes are complex cells that are increasingly implicated as playing essential roles in normal CNS function and the response to CNS disease (Barres 2008; Freeman 2010). Astrocytes *in vitro* can produce a wide variety of molecules with pro- or anti-inflammatory effects and astrocytes *in vivo* can regulate CNS inflammation through both pro- and anti-inflammatory mechanisms (Sofroniew 2009). While it was known that astrocytes express estrogen receptors (Giraud et al. 2010) and that estrogen treatment decreases expression of various pro-inflammatory molecules made by astrocytes *in vitro* (Cerciat et al. 2010), until now a direct effect of estrogen on ERα bearing astrocytes *in vivo* had not been addressed. Our data identify astrocytes as novel and critical effector cells of estrogen-mediated neuroprotective effects during CNS inflammation and have implications for understanding the pathophysiology of sex hormone effects in diverse CNS disorders. Rather than by acting directly through neurons, our findings show that estrogen signaling via ERα can significantly attenuate an inflammatory neurodegenerative process by acting through astrocytes, pointing towards novel cell-non-

autonomous mechanisms of neuroprotection in the CNS.

MATERIALS and METHODS

Animals

All mice were on a C57BL/6 background achieved by at least 10 generations of back crossing. Astrocyte-ERα-CKO were generated by crossing mice of mGFAP-Cre line 73.12 (Herrmann et al. 2008) with mice carrying an ERα gene in which exon 3 was flanked by loxP sites ($ER\alpha^{flox/flox}$) were the generous gift of Professor Pierre Chambon (Strasbourg) (Dupont et al. 2000). Neuron-ERα-CKO were generated by crossing rNSE-Cre mice (Forss-Petter et al. 1990; Kwon et al. 2006) with mice carrying an ERα gene in which exon 3 was flanked by loxP sites ($ER\alpha^{flox/flox}$) (Dupont et al. 2000). Animals were maintained under standard conditions in a 12-hour 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 male and female 1-3 mouse pups as described previously (Hamby et al. 2006). Cortices were dissected and dissociated aseptically and cells plated in 35 mm 6-well plates (1.2-1.6 hemispheres/12 ml/plate; Falcon Primaria, BD Biosciences). Plating media consisted of L-glutamine-free DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 10% iron-supplemented calf serum (CS; 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 days), cultures were treated with 8 μM cytosine β-D-arabinofuranoside (5-6 days) to antagonize microglial growth. Thereafter, cultures were maintained in L-glutamine-free DMEM containing 10% CS, 50 IU/ml penicillin and 50 μg/ml streptomycin and 2 mM L-glutamine. One day prior to experimentation, cultures were treated

with 75 mM L-leucine methyl ester (LME; 60-90 min) to further reduce microglia contamination (Hamby et al. 2006). All cultures were grown and maintained at 37°C in a humidified incubator containing 6% CO_2.

Reverse transcriptase-polymerase chain reaction (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 (no 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/30sec, 58°C/45sec, 72°C/60sec) and β-actin was amplified for 23 cycles (94°C/30sec, 65°C/45sec, 72°C/60sec). 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 day-old of wild type WT and CKO mice as previously described (Bondar et al. 2009). 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, Eugene, OR) and a fire-polished glass Pasteur pipette. Cultures were maintained at 37°C with 5% CO_2 and grown in DMEM/F12 media (Mediatech, Herndon, VA) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) and 1% penicillin-streptomycin

(PS; Mediatech, Herridon, VA) for 14-20 days. Once grown to confluency, astrocyte cultures were were used for the experiment.

Ovary tissue and astrocyte cells were homogenized in RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA) 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 amount of protein were combined with sample buffer, boiled, separated by 10% sodium dodecyl sulfate polyacrylamide gel, and then transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ). For the detection primary anti-Estrogen Receptor α (1:1000, Upstate Biotechnology, Inc., Temecula, CA) and secondary donkey anti-rabbit IgG (H+L) antibodies (1:5000, Jackson ImmunoResearch, West Grove, PA) were used. Bands were visualized using an Enhanced Chemiluminescence (ECL™) kit and ECL Hyperfilm™ (GE Healthcare, Piscataway, NJ). For determination of the molecular weight of the proteins, samples were run with Biotinylated Protein Ladder (Cell Signaling Danvers, MA). Images were processed using Adobe Photoshop.

Adoptive EAE and Hormone Manipulations

C57BL/6 donor animals were immunized subcutaneously with Myelin Oligodendrocyte Glycoprotein, amino acids 35-55 (200 μg/animal, American Peptides) emulsified in Complete Freund's Adjuvant (CFA), supplemented with *Mycobacterium Tuberculosis H37Ra* (200 μg/animal, Difco Laboratories), over four sites drained by inguinal and auxiliary lymph nodes in a total volume of 0.1 mL/mouse. Immunized mice had lymph node cells (LNCs) cultured in 24-well plates at a concentration of $3x10^6$ 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-hours. On the third day of culture, LNCs were washed with 1X PBS and

each recipient mouse received 3x10^7 cells in 0.3ml ice-cold PBS by intraperitoneal injection. Recipient female C57Bl/6 WT and CKO mice had been gonadectomized at 4 weeks of age, and had EAE induced by adoptive transfer at 8 weeks 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/day or vehicle diluted with 10% molecular-grade ethanol (EM Sciences) and 90% Miglylol 812N liquid oil (Sasol North America) beginning seven days before adoptive transfer. This dose of PPT has been previously established (Morales 2006). Animals were monitored daily for EAE signs based on a standard EAE 0-5 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 1X PBS for 20-30 minutes, followed by 10% formalin for 10-15 minutes. Spinal cords were dissected and submerged in 10% formalin overnight at 4°C, followed by 30% sucrose for 24 hours. Spinal cords were cut in thirds and embedded in optimal cutting temperature compound (Tissue Tek) and frozen at -80°C. 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 0.1M PBS with 1% sodium azide in 4°C until immunohistochemistry.

Immunohistochemistry

Prior to histological staining, 40 μm thick free-floating sections were thoroughly washed with 0.1M PBS to remove residual sodium azide. For tissues to be treated with diaminobenzidine (DAB), sections were permeabilized with 0.5% Triton X-100 in 0.1M TBS and 10% normal goat serum (NGS) for 60 minutes at RT. 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), anti-glial 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 minutes in 0.1M TBS. Tissues were labeled with secondary antibodies conjugated to Cy5 (Vector Labs and Chemicon) for 1 hour 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 semi-dry, and cover slipped in fluoromount G (Fisher Scientific). DAB sections were dried over night and then dehydrated in 70%, 95% and 100% ethonal, followed by 5 minutes of Ctitrasolve and coverslipped with Permount (Fisher). IgG-control experiments were performed for all primary antibodies, and only non-immunoreactive tissues under these conditions were analyzed. IHC for ERα was followed directly as previously described (Giraud et al. 2010).

Quantification

To quantify the astrocyte culture PCR and WB results, we quantified the optical density from the gel bands using ImageJ Software v1.30, downloaded from the NIH website (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-T5 level or three hippocampal sections from each mouse were captured under microscope at 10X or 40X 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. In order 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 40X 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 40x 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, Mannheim, Germany) or a fluorescence microscope (BX51WI; Olympus, Tokyo, Japan) 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, San Jose, CA) and Microsoft PowerPoint. DAB sections were examined at the light level at 40x (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 1x PBS for 20-30 minutes. Brains were dissected and spinal cords were flushed with 1x 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 minutes at 37°C, then passed through a wire mesh screen, followed by 100 μm, 70 μm, 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 minutes centrifugation at 1800 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 minutes 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 minutes 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).

FIGURES and LEGENDS

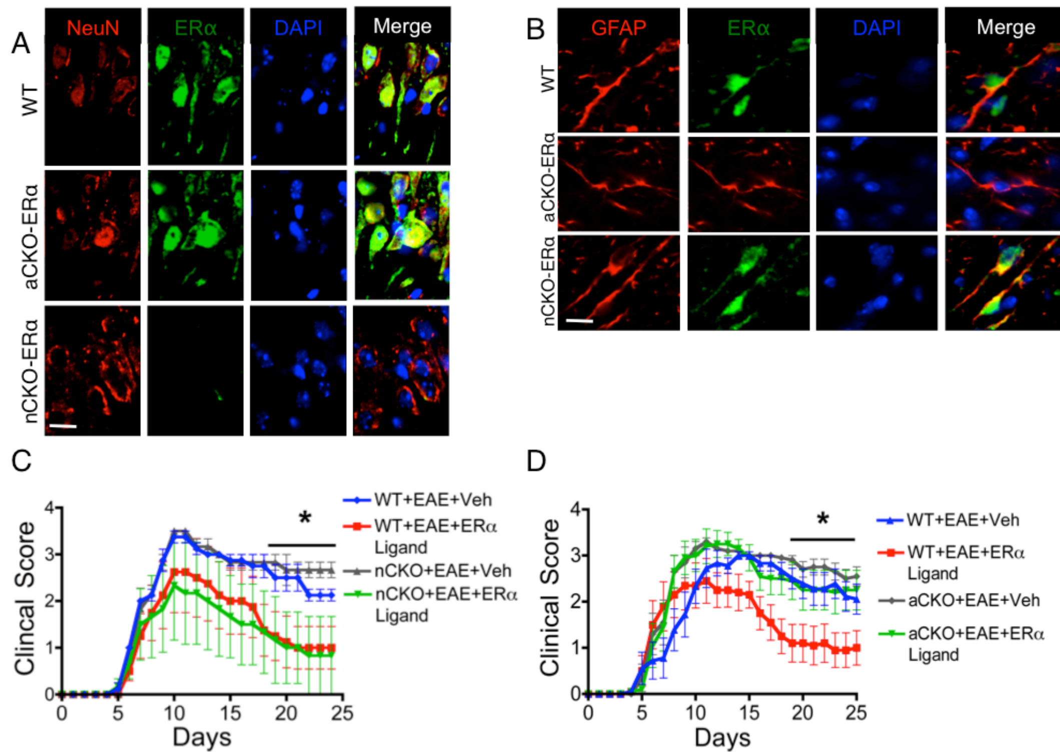

Figure 1. Verification of gene deletion specificity in astrocyte-ERα-CKO (aCKO-ERα) and neuronal-ERα-CKO (nCKO-ERα) mouse models; and EAE clinical disease severity scores showing that protective effects of ERα ligand require ERα in astrocytes, but not neurons. (A) Immunohistochemistry shows ERα co-localized with NeuN and DAPI in WT and aCKO-ERα mice with EAE, but not in nCKO-ERα mice with EAE. (B) ERα is co-localized with GFAP and DAPI in WT and nCKO-ERα mice with EAE, but not in aCKO-ERα mice with EAE. Scale bars =15μm. (C) WT and nCKO-ERα mice with EAE and given ERα ligand both had significantly better clinical scores as compared to WT and nCKO-ERα mice with EAE and given vehicle. n = 6 per group. (D) Only WT mice, but not aCKO-ERα mice, with EAE and given ERα ligand had significantly better clinical scores compared to WT or aCKO-ERα mice with EAE and given vehicle. n = 12 per group. * $p<0.05$ (repeated measures ANOVA with post hoc Bonferroni pair wise analysis).

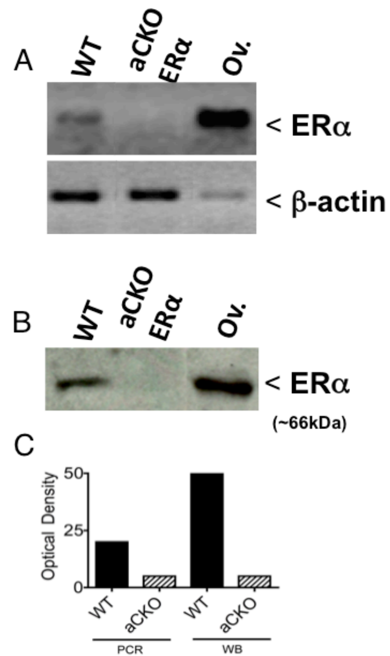

Figure 2. RT-PCR and western blot (WB) evidence that ERα is deleted from astrocyte-ERα-CKO (aCKO-ERα) astrocytes. (A) RT-PCR and (B) WB show that ERα is essentially absent in primary astrocyte cultures from aCKO-ERα mice compared with WT mice. Ovary (OV.) was used as a positive control. (C) Optical density of bands. n=4 per group.

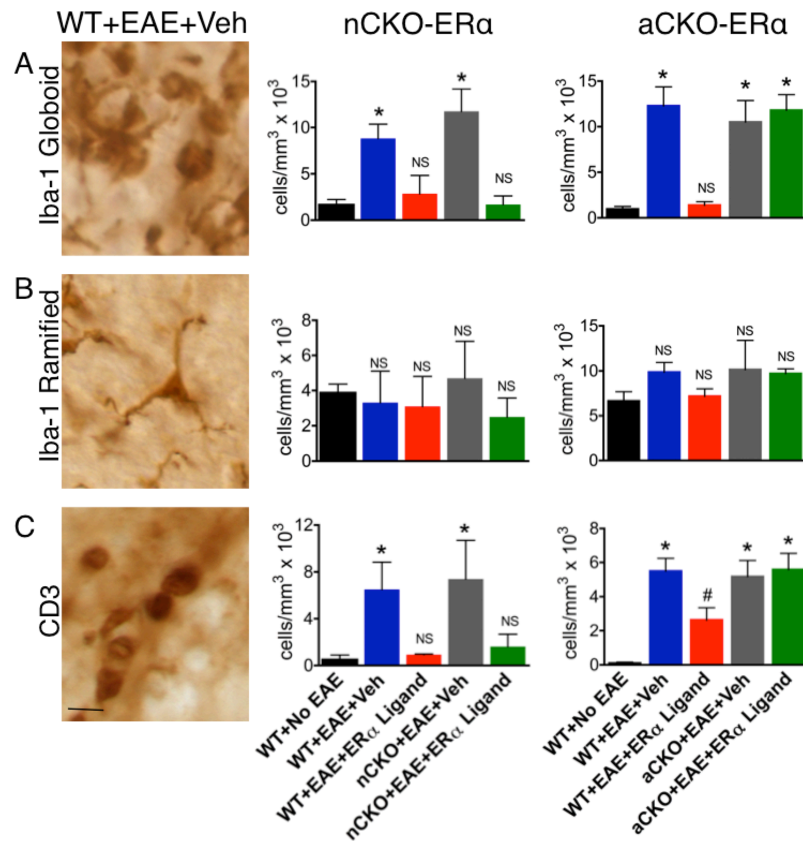

Figure 3. Immunohistochemical evidence that ERα is required in astrocytes, but not neurons, to reduce numbers of Iba-1 globoid macrophages and CD3 T- cells in dorsal column white matter. (A) Iba-1 globoid macrophages were significantly reduced in WT and neuronal-ERα-CKO (nCKO-ERα) mice with EAE treated with ERα ligand, but not in astrocyte-ERα-CKO (aCKO-ERα) mice with EAE treated with ERα ligand. (B) Iba-1 ramified microglia exhibited no significant difference in number across all experimental groups. (C) CD3 T-cells were reduced in WT and nCKO-ERα mice with EAE treated with ERα ligand, but not in aCKO-ERα mice with EAE treated with ERα ligand. Scale bar=15μm. n=6 per group. * = $p<0.05$, NS=not significant versus WT+No EAE; #=$p<0.05$ versus WT+EAE+Veh, aCKO+EAE+Veh or aCKO+EAE+ERα ligand (ANOVA with post hoc Bonferroni pair wise analysis).

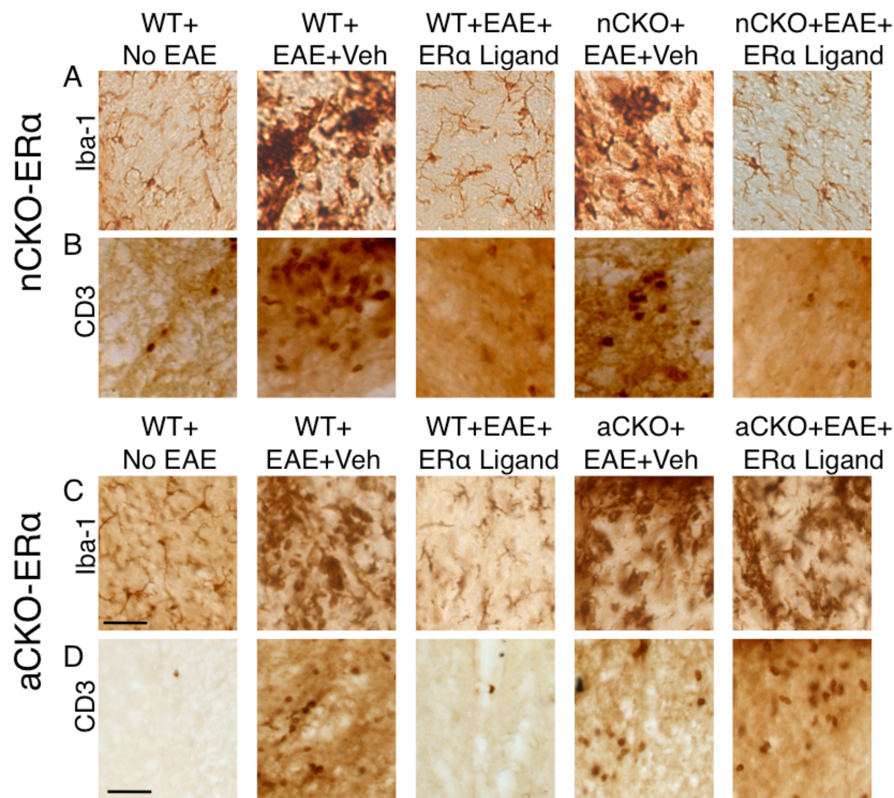

Figure 4. Immunohistochemical evidence that ERα is required in astrocytes, but not neurons, to reduce Iba-1 globoid macrophage and CD3 T-cell inflammation in dorsal column white matter of thoracic spinal cord. (A/B) Iba-1 globoid macrophages and CD3 T-cells are increased in WT mice and neuronal-ERα-CKO (nCKO-ERα) with EAE as compared with WT mice without EAE. Treatment with ERα ligand ameliorates these increases in both WT mice and in nCKO-ERα mice with EAE. (C/D) Iba-1 globoid macrophages and CD3 T-cells are increased in WT mice and astrocyte-ERα-CKO (aCKO-ERα) with EAE as compared with WT mice without EAE. Treatment with ERα ligand ameliorates these increases in WT mice but not in aCKO-ERα mice with EAE. Scale bar=25μm.

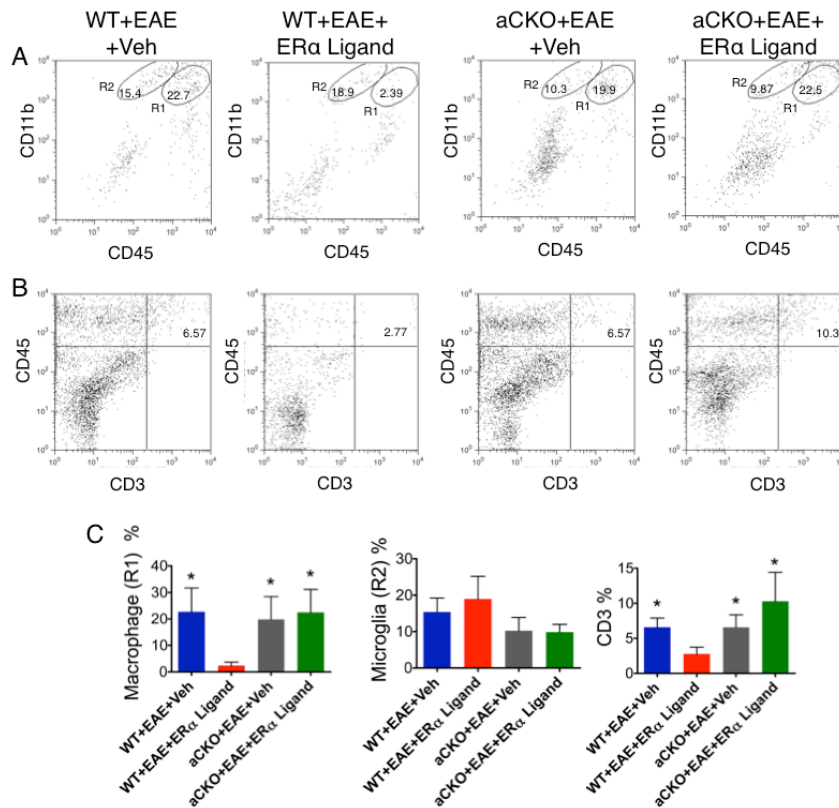

Figure 5. Flow cytometry evidence that ERα is required in astrocytes, but not neurons, to reduce macrophage and T-cell inflammation. (A/C) Macrophages (R1) ($CD11b^{hi}/CD45^{hi}$) and (B/C) T-cells ($CD45^{hi}/CD3^{hi}$) were significantly reduced in WT, but not astrocyte-ERα-CKO (aCKO-ERα) mice, treated with ERα ligand via flow cytometry from the CNS. (A/C) There were no significant differences among all groups in numbers of microglia (R2) ($CD11b^{hi}/CD45^{int}$). n=5 per group. * $p<0.05$, NS=not significant versus WT+EAE+ERα ligand (ANOVA with post hoc Bonferroni pair wise analysis).

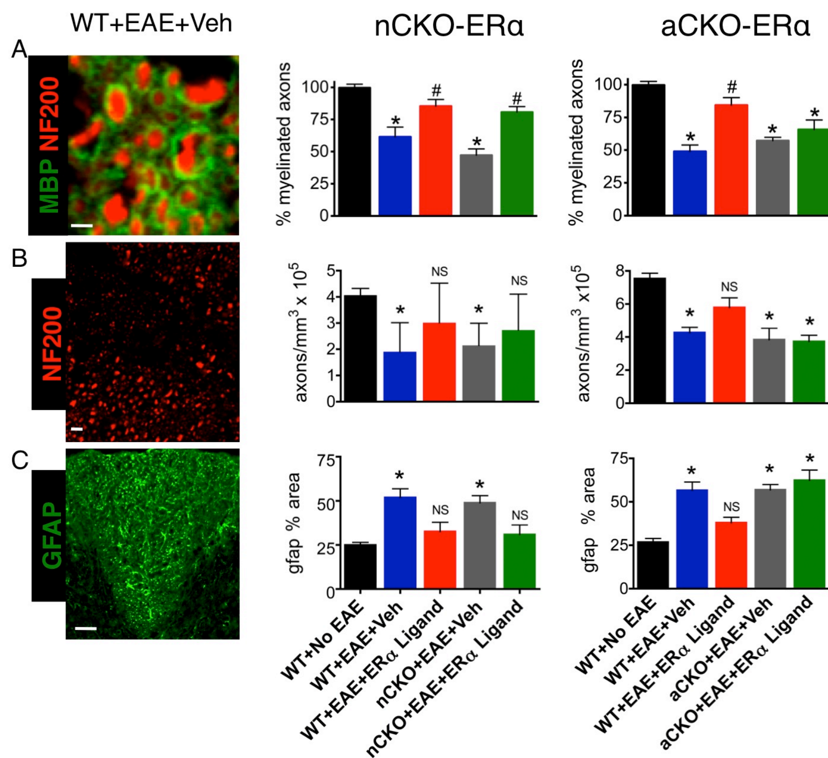

Figure 6. Immunohistochemical evidence that ERα is required in astrocytes, but not neurons, to protect against demyelination, axonal loss and reactive astrogliosis. (A) Numbers of NF200 positive axons fully encased by myelin basic protein (MBP) were significantly reduced in WT mice with EAE; and treatment with ERα ligand ameliorated demyelination in WT and neuronal-ERα-CKO (nCKO-ERα) mice, but not astrocyte-ERα-CKO (aCKO-ERα) mice, with EAE. Scale bar = 5μm. (B) Numbers of NF200 positive axons were significantly reduced in WT mice with EAE; and treatment with ERα ligand ameliorated axonal loss in WT and neuronal-ERα-CKO (nCKO-ERα) mice, but not astrocyte-ERα-CKO (aCKO-ERα) mice, with EAE. Scale bar = 20μm. (C) GFAP stained area was significantly increased in WT mice with EAE; and treatment with ERα liga nd ameliorated this increase in WT and nCKO-ERα mice, but not aCKO-ERα mice, with EAE. Scale bar =40μm. n=6 per group. * = $p<0.05$, NS=not significant versus WT+No EAE (ANOVA with post hoc Bonferroni pair wise analysis).

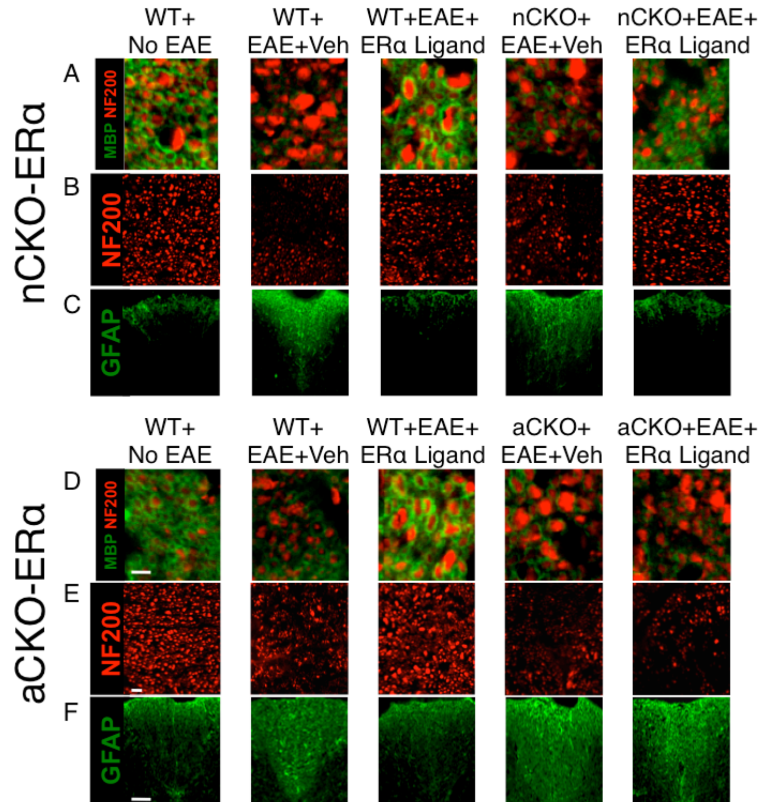

Figure 7. Immunohistochemical evidence that ERα is required on astrocytes, but not neurons, to protect against axonal loss and gliosis in dorsal column white matter of thoracic spinal cord. (A/D) MBP exhibits a patchy reduction in all groups with EAE. Scale bar=60μm (B/E) NF200 positive axons exhibit patchy reductions in numbers in WT mice with EAE. Treatment with ERα ligand ameliorated axonal loss in WT and neuronal-ERα-CKO (nCKO-ERα) mice, but not astrocyte-ERα-CKO (aCKO-ERα) mice, with EAE. Scale bar=30μm. (C/F) GFAP staining increased in intensity and area in WT mice with EAE. Treatment with ERα ligand ameliorated this increase in WT and nCKO-ERα mice, but not aCKO-ERα mice, with EAE. Scale bar=30μm.

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CHAPTER IV

Estrogen Signaling through Estrogen Receptor Alpha but not Estrogen Receptor Beta on Astrocytes Decreases Astrocyte Levels of Proinflammatory Chemokines during Experimental Autoimmune Encephalomyelitis

INTRODUCTION

Previous studies showed that treatment with either estrogen receptor alpha (ERα) ligand or estrogen receptor beta (ERβ) ligand ameliorated EAE, as well as prevented demyelination and axonal loss (Morales 2006; Du et al. 2010; Spence et al. 2011; Crawford et al. 2010; Tiwari-Woodruff et al. 2007). However, ERα ligand and ERβ ligand effects in EAE exhibit important differences. Previous data showed that ERα ligand treatment decreased the amount of immune cell infiltration into the CNS while ERβ ligand treatment did not (Tiwari-Woodruff et al. 2007). However, ERβ ligand treatment was able to qualitatively affect CNS infiltration as well as promote remyelination (Crawford et al. 2009; Du et al. 2010). Together, these observations suggest that the differences between the two ER ligands is not only due to different pharmacological effects on each receptor, but that each ligand may have different cellular targets.

Regarding cellular targets of estrogen treatments, previous work from our laboratories demonstrated that astrocytes, but not neurons, are the target of ERα ligand's protective effects during EAE (Spence et al. 2011), while neither astrocytes nor neurons are the target of ERβ ligand's protective effects. Given the differential neuroprotective effects of ERα ligand treatment versus ERβ ligand treatment on astrocytes, as well as on T-cell and macrophage inflammation, we looked for molecules within astrocytes that were affected by signaling through ERα, but not ERβ. We found that ERα ligand treatment, but not ERβ ligand treatment, decreased expression of the chemokines CCL2 and CCL7 by astrocytes in EAE. Together our data show that neuroprotection in EAE mediated via ERβ signaling does not require ERβ on astrocytes, whereas neuroprotection in EAE mediated via ERα signaling requires ERα on astrocytes and reduces astrocyte expression of chemokines that contribute to CNS inflammation. These findings reveal important cellular differences in the neuroprotective mechanisms of estrogen signaling through ERα and ERβ in EAE.

RESULTS

ERα Ligand Treatment Decreases CCL2 Expression in Astrocytes in EAE. Since we had shown a distinction between ERα and ERβ ligand's ability to affect T-cell and macrophage inflammation in the CNS, we used this selectivity to ascertain which small inflammatory molecules within astrocytes that ERα ligand, but not ERβ ligand, might regulate. One molecule of interest was chemokine (C-C motif) ligand 2 (CCL2), previously known as monocyte chemotactic protein 1 (MCP-1). CCL2 is expressed in astrocytes and is known to correlate with a higher disease severity in various CNS injury models (Hamby et al. 2012; Brambilla 2005; Carrillo-de Sauvage et al. 2012; Conductier et al. 2010). In MS tissue, CCL2 is expressed in astrocytes surrounding active and chronic lesions (McManus et al. 1998; Van Der Voorn et al. 2010; Simpson et al. 1998). In animal models CCL2, and its receptor chemokine (C-C motif) receptor 2 (CCR2), KO mice are resistant to EAE (Huang et al. 2001; Izikson et al. 2000). This resistance could be due to a decrease in macrophage recruitment into the CNS in these CCL2 KO mice (Huang et al. 2001). Here, we demonstrated that CCL2 is expressed within reactive astrocytes and immune cells in the spinal cord of EAE mice (Fig. 1 A/B/C). Treatment with ERα ligand *in vivo* was able to reduce CCL2 expression in reactive astrocytes in WT mice as compared to vehicle. Interestingly, ERα ligand treatment lost its ability to reduce CCL2 expression in reactive astrocytes in astrocyte-CKO-ERα mice with EAE, thereby demonstrating that ERα ligand does indeed act on astrocytes *in vivo* to reduce CCL2 expression (Fig. 1D). In contrast, *in vivo* treatment with ERβ ligand was not able to reduce CCL2 expression in reactive astrocytes in any of the groups with EAE (Fig. 1E). Together this demonstrates the selectivity of ligation of ERα, but not ERβ, on astrocytes in decreasing CCL2 expression during EAE.

ERα Ligand Treatment Decreases CCL7 Expression in Astrocytes in EAE. Given our CCL2 data, we investigated other chemokines expressed by astrocytes that could be

differentially affected by ERα versus ERβ ligation during EAE. Our next molecule of interest was CCL7*,* previously known as monocyte chemotactic protein 3 (MCP-3). CCL7 is expressed in astrocytes and previous research showed that CCL7 expression directly correlated with CCL2 expression in astrocytes cultures (Hamby et al. 2012; Thompson & Van Eldik 2009). In MS post-mortem tissue, CCL7 expression is expressed in astrocytes and surrounds MS lesions(McManus et al. 1998). Furthermore, CCL7 expression has also been detected in the spinal cords of EAE animals (Adzemovic et al. 2012). Therefore we examined CCL7 expression within reactive astrocytes and saw a similar expression pattern to that of CCL2 in astrocytes (Fig. 2A/B). However, unlike CCL2, CCL7 expression was only found in reactive astrocytes and was not detected in infiltrating immune cells (Fig. 2C). Treatment with ERα ligand *in vivo* was able to reduce CCL7 expression in reactive astrocytes in WT mice as compared to vehicle. However, ERα ligand treatment lost its ability to reduce CCL7 expression in reactive astrocytes in astrocyte-CKO-ERα mice with EAE, thereby demonstrating that ERα ligand does indeed act on astrocytes *in vivo* to reduce CCL7 expression (Fig. 2D). In contrast, treatment with ERβ ligand was not able to reduce CCL7 expression in reactive astrocytes in any of the groups with EAE (Fig. 2E) suggesting that CCL7 expression in astrocytes can be decreased by ERα, but not ERβ, ligand treatment *in vivo.* We also looked for expression of CCL5 and CCL8 but did not observe expression of these chemokines within astrocytes.

ERα Ligand Treatment Does Not Decrease Aquaporin 4 Expression in Astrocytes in EAE. Another possible small molecule expressed by astrocytes that could be responsible for the differential effects of ERα versus ERβ ligand treatment on inflammation is aquaporin-4 (AQP4). AQP4 is a water channel expressed by astrocytes and thought to play a critical role in the maintenance of the blood to brain barrier (BBB) (Verkman et al. 2011; Verkman 2009; Verkman et al. 2006). CNS diseases, such as spinal cord injury, are known to alter the expression of AQP4 (Nesic et al. 2010; Kimura et al. 2010). In EAE, AQP4 expression is

upregulated and redistributed, while AQP4 KO mice are resistant to EAE (Miyamoto et al. 2009; Li et al. 2009; Wolburg-Buchholz et al. 2009). Interestingly, treatment with estradiol had been previously shown to decrease AQP4 expression in astrocytes *in vitro* (Rutkowsky et al. 2011). Thus, we quantified the expression of AQP4 in reactive astrocytes. Our results demonstrated that AQP4 is indeed expressed within reactive astrocytes in the spinal cord of EAE mice (Fig. 3 A/B). However, AQP4 expression in astrocytes was increased in all mice with EAE regardless of genotype or treatment, suggesting that AQP4 was not altered with ERα or ERβ ligand treatment in either WT or astrocyte-CKO mice (Fig. 3 C/D). Together these data revealed that the effects of ERα ligand treatment on astrocytes *in vivo* during EAE are selective in that they affect the expression of specific chemokines such as CCL2 and CCL7, but not other immunomodulatory molecules like AQP4.

DISCUSSION

Together our findings reveal important differences in the cellular mechanisms that underlie the neuroprotective effects of estrogen signaling through either ERα or ERβ in EAE. Our findings here extend these observations by showing estrogens can exert neuroprotection in EAE through markedly different cellular mechanisms when signaling through either ERα or ERβ. Consistent with previous studies, we found that both ERα-ligand and ERβ-ligand decreased clinical disease, demyelination, and axonal loss in EAE, and that ERα-ligand, but not ERβ-ligand, significantly reduced inflammation (Tiwari-Woodruff et al. 2007; Crawford et al. 2009; Du et al. 2010; Morales 2006; Spence et al. 2011). Notably, we found that the neuroprotective effects of ERα-ligand were completely lost when ERα was selectively deleted from astrocytes, whereas selective deletion of ERβ from astrocytes had no effect on the neuroprotective effects of ERβ-ligand. Thus, the neuroprotective effects of ERα-ligand are mediated through astrocytes, whereas the neuroprotective effects of ERβ-ligand are mediated by some other cell type, perhaps by acting on microglia, dendritic cells, neurons or oligodendrocytes (Saijo et al. 2011; Du et al. 2010; Crawford et al. 2010). In addition, we show that only signaling through ERα, but not ERβ, decreased inflammation in EAE, and that this effect required ERα on astrocytes and that ERα-ligand reduced astrocyte levels of inflammatory chemokines such as CCL2 and CCL7 (Fig. 4). Together, these findings demonstrate that markedly different cellular mechanisms underlie the neuroprotective effects of signaling through ERα or ERβ.

Implications for our results are not limited to the MS model (Morissette et al. 2008). In animal models of ischemia, estradiol is known to reduce total infarct size. However, this protective effect is lost in a global ERα KO, but not in a global ERβ KO, mice treated with estradiol, demonstrating that ERα, but not ERβ, expression is necessary for estradiol neuroprotection during ischemia (Dubal et al. 2001; Dubal 2006). In another study, MPTP was given to WT as well as both global ERα KO and global ERβ KO. In the absence of exogenous estrogen administration, the level of striatal dopamine loss was greater in global ERα KO

compared to the WT and ERβ KO, suggesting that ERα is more important than ERβ in the MPTP model. However, when exogenous estradiol was given, both the ERα KO and ERβ KO groups demonstrated a decrease in dopamine, suggesting that while ERα is more important at physiological levels, ERβ can play a role at therapeutic levels(Morissette et al. 2007). While the exact mechanisms of ERα versus ERβ neuroprotection are unknown, researchers have started to uncover differential signaling events between ERα and ERβ in the brain. In further MPTP studies, ERα ligand was able to decrease IGF-1 levels as well as phosphorylation of Akt, while ERβ ligand had no effect. Furthermore, ERα ligand, but not ERβ ligand, was able to prevent a decrease in the Bcl-2:BAD protein ratio after MPTP administration (D'Astous et al. 2006). In another example, in hippocampal primary neurons, ERα ligand was not as effective as ERβ ligand in increasing the concentration of a glutamate induced intracellular calcium rise. Furthermore, while both ligands were able to increase ERK phosphorylation in these neurons, the magnitude and timing of this increase was unique to each ligand (Zhao & Brinton 2007). In another hippocampal study, treatment with ERβ ligand but not ERα ligand, increased the synaptic proteins PSD-95, synaptophysin and the AMPA- receptor subunit GluR1. This increase in synaptic proteins correlated with an improved performance in hippocampus-dependent memory tasks. These positive effects of ERβ ligand treatment were lost in the global ERβ KO model (Liu et al. 2008). Together, these findings are all consistent with the notion that ERα or ERβ signaling mediate neuroprotection via different cellular mechanisms, similar to our observations reported here.

Astrocytes have multiple functions and play complex roles in CNS function and disease (Barres 2008; Freeman 2010; Sofroniew & Vinters 2009) including MS and EAE (Chastain et al. 2011; Liedtke et al. 1998; Voskuhl et al. 2009). Astrocytes have been shown to produce a multitude of pro- and anti-inflammatory molecules that can alter CNS inflammation (Hamby et al. 2012; Sofroniew 2009; Argaw et al. 2012). Given estrogens' ability to alter pro and anti-inflammatory molecules in astrocytes, (Arevalo et al. 2010; Cerciat et al. 2010; Giraud et al. 2010) and given our observed differences in effects on inflammation between ERα and ERβ

ligand treated EAE mice, we investigated inflammatory molecules expressed by astrocytes that could be responsible for ERα ligand mediated disease protection during EAE. Previous research showed that estrogen treatment *in vivo* correlated with a decrease in CCL2 expression in astrocytes during EAE, but whether estrogen acted directly or indirectly on astrocytes remained unknown. Further *in vitro* analysis, using astrocyte cultures, showed that this effect most likely occurred through ERα (Giraud et al. 2010). Our results extend these results by demonstrating for the first time *in vivo* that CCL2 and CCL7 are decreased upon ligation of ERα, but not ERβ, on astrocytes in EAE. Another astrocyte molecule reported to influence inflammation is AQP4. Previous *in vitro* data reported that estrogen was able to decrease AQP4 expression in an animal model of ischemia (Rutkowsky et al. 2011). Interestingly, our *in vivo* data showed that neither ERα ligand or ERβ ligand significantly altered AQP4 expression on astrocytes. These differences could be due to the fact that we used ER specific ligands as opposed to estradiol which can act on both nuclear and membrane ERα and ERβ. Alternatively, there may be disease related differences since the previous reports used an animal model of ischemia, while we focused on an animal model of MS. To fully elucidate the role that astrocyte expression of CCL2 and CCL7 play in inflammation, studies using an astrocyte CKO of CCL2 (Ge et al. 2009) or CCL7 or both CCL2 and CCL7 are warranted.

MATERIALS AND METHODS

Animals

All mice were on a C57BL/6 background achieved by at least 10 generations of back crossing. Astrocyte-ERα-CKO were generated by crossing mice of mGFAP-Cre line 73.12 (Herrmann et al. 2008) with mice carrying an ERα gene in which exon 3 was flanked by loxP sites ($ER\alpha^{flox/flox}$) were the generous gift of Professor Pierre Chambon (Strasbourg) (Dupont et al. 2000). Astrocyte-ERβ-CKO were generated by crossing mice of mGFAP-Cre line 73.12 (Herrmann et al. 2008) with mice carrying an ERβ gene in which exon 3 was flanked by loxP sites ($ER\beta^{flox/flox}$) were the generous gift of Professor Pierre Chambon (Strasbourg) (Dupont et al. 2000). Animals were maintained under standard conditions in a 12-hour 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.

Adoptive EAE and Hormone Manipulations

C57BL/6 donor animals were immunized subcutaneously with Myelin Oligodendrocyte Glycoprotein, amino acids 35-55 (200 µg/animal, American Peptides) emulsified in Complete Freund's Adjuvant (CFA), supplemented with *Mycobacterium Tuberculosis H37Ra* (200 µg/animal, Difco Laboratories), over four sites drained by inguinal and auxiliary lymph nodes in a total volume of 0.1 mL/mouse. Immunized mice had lymph node cells (LNCs) cultured in 24-well plates at a concentration of $3x10^6$ 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-hours. On the third day of culture, LNCs were washed with 1X PBS and each recipient mouse received $3x10^7$ cells in 0.3ml ice-cold PBS by intraperitoneal injection. Recipient female C57Bl/6 WT and astrocyte-CKO mice had been gonadectomized at 4 weeks of age, and had EAE induced by adoptive transfer at 8 weeks 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/day or ERβ ligand (DPN) (Tocris) at the dose of 8 mg/kg/day or vehicle diluted with 10% molecular-grade ethanol (EM Sciences) and 90% Miglylol 812N liquid oil (Sasol North America) beginning seven days before adoptive transfer. These doses of PPT and DPN have been previously established (Tiwari-Woodruff et al. 2007).

Histological preparation

Female mice were deeply anesthetized in isoflurane and perfused transcardially with ice-cold 1X PBS for 20-30 minutes, followed by 10% formalin for 10-15 minutes. Spinal cords were dissected and submerged in 10% formalin overnight at 4°C, followed by 30% sucrose for 24 hours. Spinal cords were cut in thirds and embedded in optimal cutting temperature compound (Tissue Tek) and frozen at -80°C. 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 0.1M PBS with 1% sodium azide in 4°C until immunohistochemistry.

Immunohistochemistry

Prior to histological staining, 40 μm thick free-floating sections were thoroughly washed with 0.1M PBS to remove residual sodium azide. For tissues to be treated with diaminobenzidine (DAB), sections were permeabilized with 0.5% Triton X-100 in 0.1M TBS and 10% normal goat serum (NGS) for 60 minutes at RT. The following primary antibodies were used: anti-glial fibrillary acidic protein-GFAP at 1:40,000 (Dako), anti-anti-MCP-1 (CCL2) at 1:200 (Torrey Pines Biolabs), and CCL7 (Sigma) at 1:50. Tissues were then washed three times for 10 minutes in 0.1M TBS. Tissues were labeled with secondary antibodies conjugated to Cy5 or Cy3 (Vector Labs and Chemicon) for 1 hour for CCL2, CCL5, CCL7, CCL8, and GFAP. Fluorescent sections were mounted on slides, allowed to semi-dry, and cover slipped in fluoromount G (Fisher Scientific). IgG-control experiments were performed for all primary antibodies, and only non-immunoreactive tissues under these conditions were analyzed.

Quantification

To quantify immunohistochemical staining results, three dorsal column spinal cord cross-sections at the T1-T5 level from each mouse were captured under microscope at 10X or 40X 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 downloaded from the NIH website (http://rsb.info.nih.gov/ij). Three sections from each animal were then quantified to calculate the mean per animal. Immunohistochemical experiments were combined from three separate clinical trials. In order 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. AQP4, CCL7, and CCL2 were measured as co-expression with GFAP in ImageJ, then divided over the total amount of GFAP in each section and presented as a percent.

Microscopy

Stained sections were examined and photographed using a confocal microscope (Leica TCS-SP, Mannheim, Germany) or a fluorescence microscope (BX51WI; Olympus, Tokyo, Japan) 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, San Jose, CA) and Microsoft PowerPoint. DAB sections were examined at the light level at 40x (Nikon Alphaphot-2 YS2).

Statistical Analysis

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).

FIGURES and LEGENDS

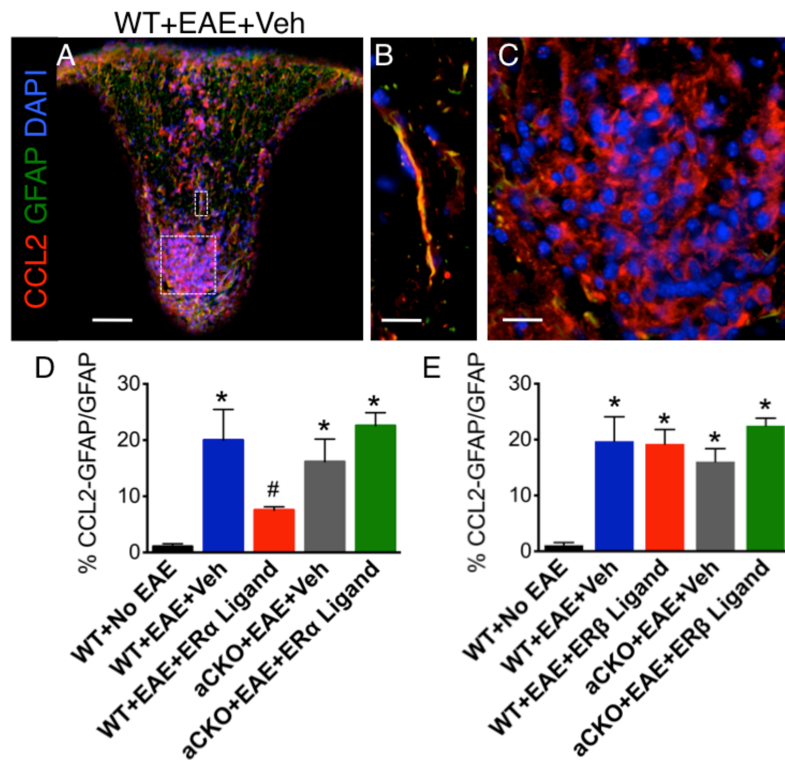

Figure 1. ERα is required on astrocytes to reduce CCL2 expression within reactive astrocytes. (A) CCL2 (red), GFAP (green) and DAPI (blue) are expressed in EAE spinal cord in a WT mouse with EAE (scale bar 120μm). (B) CCL2 is co-expressed with GFAP (scale bar, 27μm). (C) CCL2 is co-expressed with infiltrating immune cells stained with DAPI (scale bar, 27μm). (D) Treatment with ERα ligand was able to reduce co-expression of CCL2 with GFAP in WT, but not astrocyte-CKO-ERα (aCKO-ERα), mice with EAE. (E) Treatment with ERβ ligand was not able to reduce co-expression of CCL2 with GFAP in WT or astrocyte-CKO-ERβ (aCKO-ERβ) mice with EAE. n=5 per group. *P<0.05; NS, not significant vs. WT+No EAE; #P<0.05 vs. WT+EAE +Veh, aCKO+EAE+Veh, aCKO+EAE+ERα Ligand (ANOVA with post hoc Bonferroni paiwise analysis).

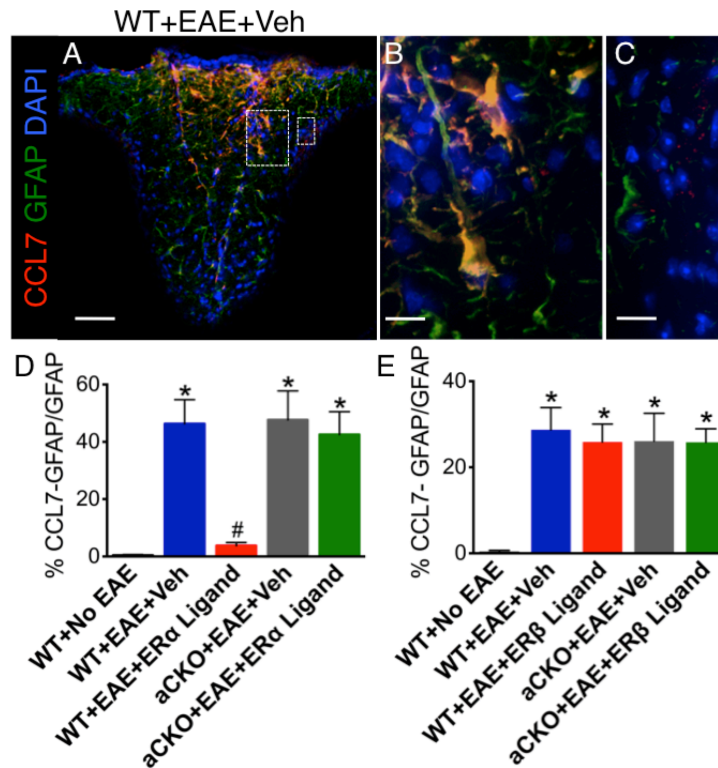

Figure 2. ERα is required on astrocytes to reduce CCL7 expression within reactive astrocytes. (A) CCL7 (red), GFAP (green) and DAPI (blue) are expressed in EAE spinal cord in a WT mouse with EAE (scale bar 120μm). (B) CCL7 is co-expressed with GFAP (scale bar, 27μm). (C) CCL7 is not co-expressed in infiltrating immune cells (DAPI, scale bar 27um). (D) Treatment with ERα ligand was able to reduce co-expression of CCL7 with GFAP in WT, but not astrocyte-CKO-ERα (aCKO-ERα), mice with EAE. (E) Treatment with ERβ ligand was not able to reduce co-expression of CCL7 with GFAP in WT or astrocyte-CKO-ERβ (aCKO-ERβ) mice with EAE. n=5 per group. *P<0.05; NS, not significant vs. WT+No EAE; #P<0.05 vs. WT+EAE+Veh, aCKO +EAE+Veh, aCKO+EAE+ERα Ligand (ANOVA with post hoc Bonferroni paiwise analysis).

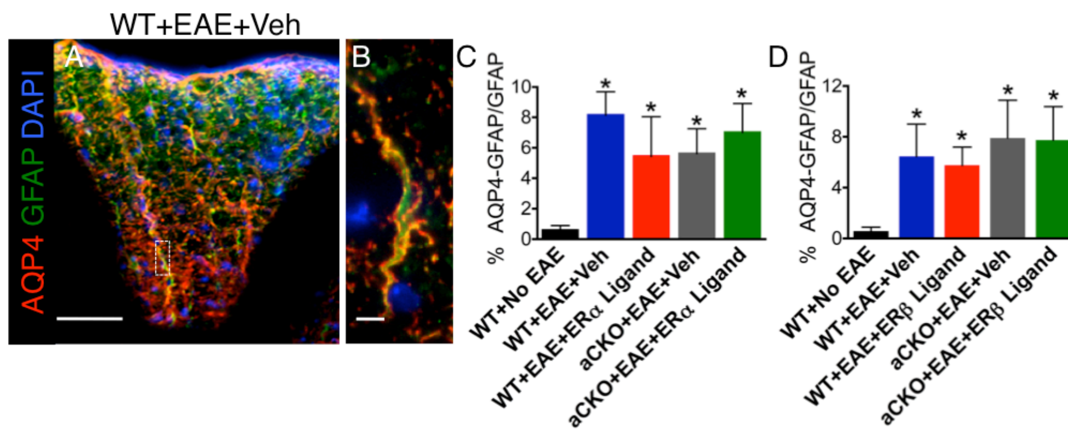

Figure 3. AQP4 expression within reactive astrocytes is increased in all groups with EAE, regardless of genotype or treatment. AQP4 (red), GFAP (green) and DAPI (blue) are expressed in EAE spinal cord in a WT mouse with EAE (scale bar 145µm). (B) AQP4 is co-expressed with GFAP (scale bar, 8µm). (C/D) AQP4 co-expression with GFAP is increased in all groups with EAE, regardless of genotype or treatment. n=5 per group. *P<0.05 vs. WT+No EAE (ANOVA with post hoc Bonferroni paiwise analysis).

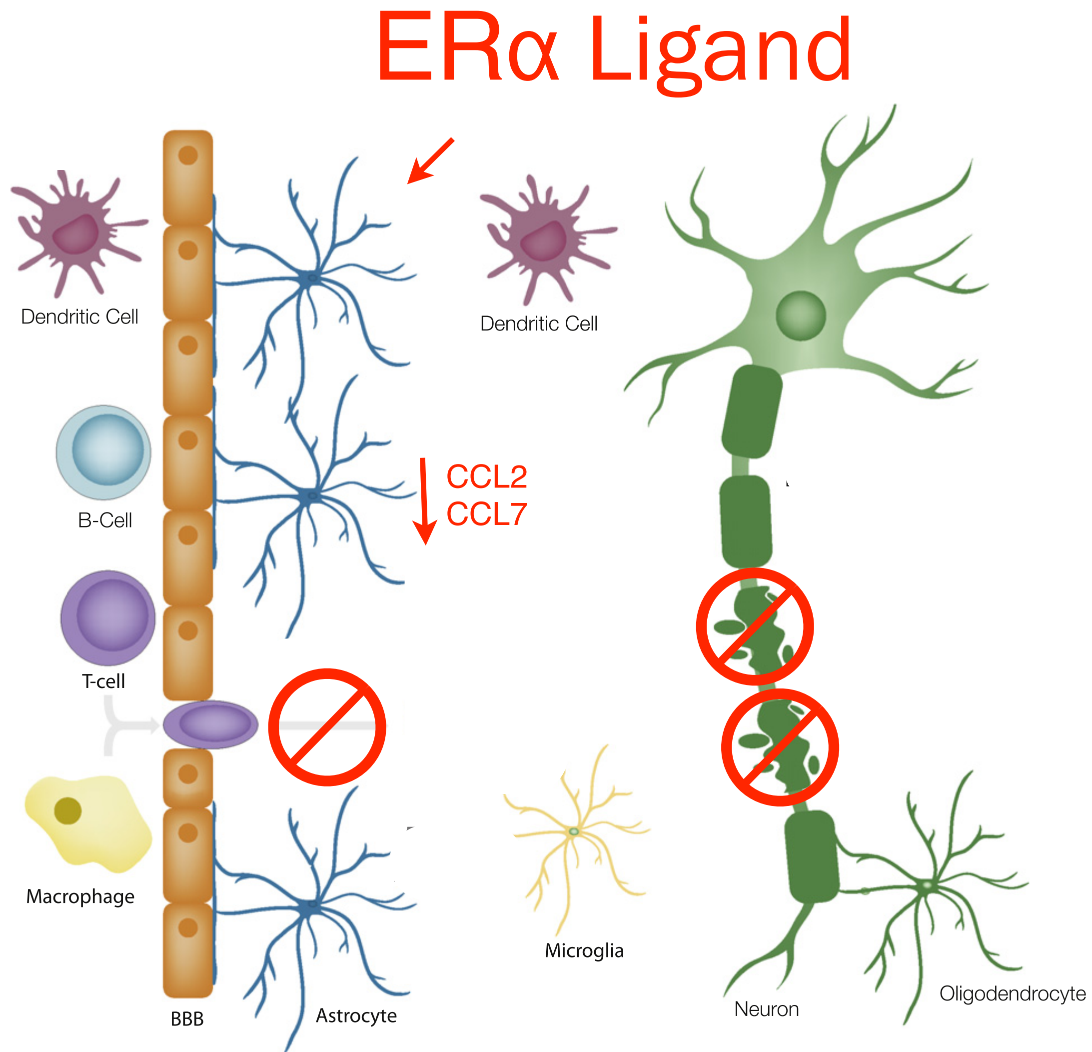

Figure 4. ERα ligand acts on ERα on astrocytes to decrease CCL2 and CCL7 production by astrocytes. This correlates with a decrease of T-cell and macrophage infiltration into the CNS perhaps in turn leading to a decrease in demyelination and axonal loss in the EAE model.

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