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ORIGINAL ARTICLE

Gender Differences in Multiple Sclerosis: Induction of Estrogen Signaling in Male and Progesterone Signaling in Female Lesions

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Abstract

The basis of gender differences in the prevalence and clinical progression of multiple sclerosis (MS) is not understood. Here, we identify gender-specific responses in steroid synthesis and signaling in the brains of MS patients as possible contributors to these differences. We investigated gene expression changes in these pathways and of inflammatory cytokines in MS lesions and normal-appearing white matter (NAWM) of male and female patients (n = 21) and control NAWM (n = 14) using quantitative polymerase chain reaction (25 MS lesions, 21 MS NAWM, and 14 control NAWM) and immunohistochemistry (3–4 sections per group). In MS lesions in males, there was local upregulation of aromatase (an enzyme involved in estrogen biosynthesis), estrogen receptor-β (ERβ), and tumor necrosis factor (TNF) mRNA; whereas in females, there was local upregulation of 3β-hydroxysteroid-dehydrogenase, a progesterone synthetic enzyme, and of progesterone receptor. Astrocytes in the rim and center of MS lesions were found to be the primary source of steroidogenic enzyme and receptor expression. Aromatase and ERα mRNA levels were positively correlated with that of TNF in primary cultures of human microglia and astrocytes; TNF caused increased ERα, suggesting that inflammatory signals stimulate estrogen signaling in this cell type. Together, these findings suggest that there are gender differences in the CNS of MS patients that may affect lesion pathogenesis, that is, in males, estrogen synthesis and signaling are induced; whereas in females, progestogen synthesis and signaling are induced. These differences may represent contributing factors to gender differences in the prevalence and course of MS.

Key Words: Gene expression, Multiple sclerosis, Neurosteroids, Postmortem human brain, Sex differences.

INTRODUCTION

There are differences in the prevalence and clinical courses of multiple sclerosis (MS) patients, but the mechanisms for these differences are not understood. The prevalence of MS is at least 2 times higher in women than in men. Women more frequently develop a benign relapsing-remitting type of MS, whereas the disease develops more often as a progressive course with more severe disability and shorter duration until death in men (1–3). Men are more prone to develop less inflammatory, but more neurodegenerative, lesions characterized as ''black holes'' in brain magnetic resonance imaging scans (4). These clinical and radiological gender differences suggest an influence of sex hormones in MS pathology.

Recently, we reported the first evidence of changes in hormone receptor expression in MS (5).

We showed that estrogen receptor-α (ERα) mRNA is increased in active and inactive MS lesion centers and inactive lesion borders (5), raising the possibility of a role for estrogens in modulating pathologic processes in MS. Indeed, in recent MS clinical trials, estrogen treatment decreased the number and volume of lesions in relapsing-remitting female patients; whereas in men, testosterone treatment led to a slowing of brain atrophy (6). In further support of the protective action of steroids in MS, the incidence of MS relapse decreases during pregnancy and increases again postpartum, when estrogen and progesterone levels rapidly decrease (2, 7).

In addition to being a target for sex steroids released by peripheral steroidogenic tissues, the CNS also produces steroids, that is, ''neurosteroids,'' de novo (8). Substantial evidence from in vivo and in vitro studies indicates that neurosteroids protect the CNS against inflammation and neurodegeneration (9–11) by neurotrophic, neuroprotective, and anti-inflammatory factors (12–15). Highly relevant for MS, estrogens, progesterone, and allopregnanolone all strongly promote myelination in mice and rats (9). Furthermore, progesterone and allopregnanolone may also promote proliferation of neural progenitors via $GABA_A$ receptors (16). Importantly, Noorbakhsh et al (17) showed that synthesis of allopregnanolone is impaired in normal-appearing white matter (NAWM) of MS patients.

In the present study, we explored the possibility that altered neurosteroid synthesis in the CNS modulates different aspects of MS pathology, such as inflammation, demyelination, and neurodegeneration in a gender-dependent manner. We analyzed the steroid synthetic pathways and the inflammatory

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mediators interleukin-1β (IL-1β), IL-6, and tumor necrosis factor (TNF) (18) in chronic active and inactive MS lesions, MS NAWM, and control (CTR) NAWM from females and males. We observed important sex differences in estrogen and progesterone synthesis and signaling in MS lesion and perilesional NAWM and show that these changes may be induced by increased inflammatory cytokine expression.

MATERIALS AND METHODS

Subjects

Postmortem material and clinical information were provided by the Netherlands Brain Bank (NBB). The NBB obtained permission from its donors for brain autopsy and the use of tissue and clinical information for research purposes.

TABLE 1. Clinicopathologic Data on Multiple Sclerosis Patients and Controls

Code	Age, years	Sex	MS Duration, years	CSF pH	PMD (hours:minutes)	MS Lesion Isolated	MS Type	Cause of Death
MS1	52	F	12	6.10	8:25	CA	SP	Pneumonia
MS2	48	F	9	6.55	8:10	CA	SP	Legal euthanasia
MS3	72	F	14	6.85	12:00	CI	SP	Pneumonia
MS4	49	F	31	6.43	5:45	CI	SP	Breast carcinoma
MS5	41	F	11	6.83	8:25	CA	SP	Natural death
MS6	78	F	30	6.32	11:10	CI	SP	Dehydration
MS7	66	F	23	6.18	6:00	CA/CI	SP	Unknown
MS8	78	F	24	6.50	10:00	CI	PP	Legal euthanasia
MS9	81	F	57	7.18	7:17	CI	SP	Sepsis
MS10	59	F	25	7.08	4:45	CA/CI	SP	Legal euthanasia
MS11	57	F	27	6.05	8:40	CA	SP	Urosepsis
	Mean ± SE, 62.9 ± 4.6					F total lesions, n = 6 CA/7CI		
MS12	43	M	17	6.48	8:30	CA	SP	Pneumonia
MS13	56	M	21	6.65	8:00	CA/CI	SP	Pneumonia
MS14	66	M	n.a.	6.29	7:45	CI	n.a.	Sepsis
MS15	47	M	7	6.20	7:15	CA/CI	SP	Urosepsis
MS16	50	M	17	6.63	5:25	CI	PP	Lung carcinoma
MS17	49	M	26	6.26	8:00	CA	SP	Pneumonia
MS18	55	M	32	6.70	6:20	CA	SP	Urosepsis
MS19	66	M	26	6.31	7:30	CI	PP	Ileus, urosepsis
MS20	61	M	18	6.88	9:15	CA	SP	Legal euthanasia
MS21	75	M	55	6.53	7:45	CI	SP	Legal euthanasia
	Mean ± SE, 56.8 ± 3.8					M total lesions, n = 6 CA/6CI		
CTR1	60	F	—	6.60	8:25	—	—	Lung carcinoma
CTR2	78	F	—	6.60	7:30	—	—	Emphysema
CTR3	65	F	—	6.94	12:50	—	—	Cardiac arrest
CTR4	41	F	—	n.a.	13:30	—	—	Adenocarcinoma
CTR5	52	F	—	7.16	6:50	—	—	Leiomyosarcoma
CTR6	81	F	—	7.16	6:40	—	—	Carcinoma
CTR7	50	F	—	6.98	4:10	—	—	Bronchocarcinoma
	Mean ± SE, 61 ± 5.9							
CTR8	60	M	—	6.71	8:50	—	—	Carcinoma
CTR9	75	M	—	6.16	7:20	—	—	Sepsis
CTR10	70	M	—	6.40	7:30	—	—	Pancreas carcinoma
CTR11	49	M	—	n.a.	9:50	—	—	Myocarditis
CTR12	51	M	—	n.a.	7:44	—	—	Ventricle fibrillation
CTR13	56	M	—	7.03	14:00	—	—	Heart failure
CTR14	62	M	—	6.36	7:20	—	—	Unknown
	Mean ± SE, 60.4 ± 4.4							
p*	0.727			0.259	0.893			

All MS cases were clinically confirmed by a neurologist and by a neuropathologist postmortem. Patients who in the last month before death were treated with drugs that may interfere with neurosteroid synthesis such as corticosteroids, indomethacin, finasteride, and hormonal therapy such as methyl progesterone (used as MS treatment) were excluded. Controls were matched for age, sex, postmortem delay, pH of cerebrospinal fluid, cause of death, and therapy as closely as possible.

*Kruskal-Wallis test for difference in given parameter between all 4 groups. No statistical differences were found between CTR and MS and between female and male groups.

CA, chronic active; CI, chronic inactive; CSF, cerebrospinal fluid; CTR, control; F, female; M, male; MS, multiple sclerosis; n.a. not available; PMD, postmortem delay; PP primary progressive; SP secondary progressive.

The NBB donor program has been approved by the Ethical Committee VU University Medical Center (Amsterdam, The Netherlands). Multiple sclerosis lesion types isolated from each patient and clinicopathologic data are presented in Table 1.

Lesion Selection and Dissection

Selection and dissection of postmortem frozen tissue containing chronic active lesions, chronic inactive lesions, and perilesional NAWM from MS patients and WM of controls are described in Figure 1.

RNA Extraction, cDNA Synthesis, and qPCR

RNA isolation and quantitative polymerase chain reaction (qPCR) methodology were performed as previously described (19). RNA purity was determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). The RNA integrity number was measured on the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). All RNA samples used in this study had RNA integrity values of at least 6. A dilution of 1:10 of the total cDNA yield was used for the qPCR experiments. *GADPH* and *TUB*α reference genes were selected by a geNorm analysis (20) and used for normalization. The relative mRNA expression of the target gene was calculated as the fraction of the absolute expression level with the average of reference gene relative values as previously described (19). Complementary DNA was synthesized using a Quantitec reverse transcription kit (cat. no. 205311; Qiagen, Benelux, Belgium). Primer pairs, amplicon lengths, and gene name abbreviations used are given in Table, Supplemental Digital Content 1, http://links.lww.com/NEN/A547.

Immunohistochemistry and Analysis of Immunohistochemical Staining

The primary antibodies used (21–26) and their dilutions are shown in Table 2. Detailed methodology is described in Materials and Methods, Supplemental Digital Content 2, http://links.lww.com/NEN/A548. The sections (1 section each from 3–4 patients per gender per group) were rated on basis of the staining (density and intensity of positive cells) by 2 independent investigators who were blinded to the subject's sex or group (MS or CTR). The rating was made at 1× magnification using a 20× objective. For a given MS lesion region (NAWM, rim, center) and for CTR WM, between 1 and 5 fields of view were randomly chosen. For each molecule of interest, pictures for each region where coded and ranked from higher to lower intensity. Scores were assigned to similar group

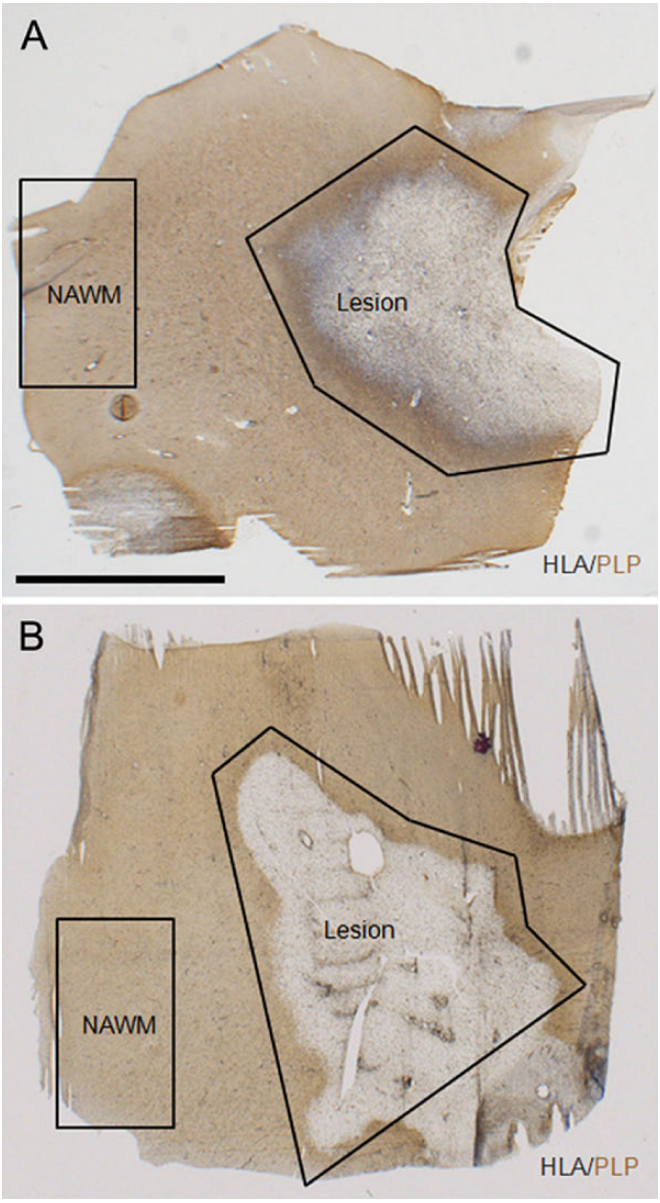

FIGURE 1. Dissection of chronic active and multiple sclerosis (MS) inactive lesions and normal-appearing white matter (NAWM) based on their histologic appearance. **(A, B)** Dissection of a chronic active lesion **(A)** and a chronic inactive lesion **(B)** with nearby NAWM. Lesions were characterized according to criteria described by van der Valk and De Groot (18) based on the histologic appearance of the myelin in myelin proteolipid protein (PLP) immunostaining and of inflammatory cells by HLA-DR-DQ (HLA) immunostaining. MS lesions with a demyelinated hypocellular core and a rim of HLA-positive foamy macrophages/microglia with reactive perilesional WM were designated as chronic active lesions **(A)**. Lesions with a hypocellular demyelinated core, few reactive HLA-positive microglia in the rim, and normally myelinated perilesional WM were designated as chronic inactive lesions **(B)**. In both MS and control donors, regions with normal myelin content with low microglia activation were regarded as NAWM. From each sample, 5 to 10 sections (60-μm thick) were cut using a cryostat, and the MS lesion including the gliotic center, rim, and perilesional area and perilesional NAWM were dissected out using a prechilled scalpel as outlined in **(A)** and **(B)**. Lesions and perilesional NAWM were isolated using Sudan black staining (which visualizes demyelination and myelin inclusions in macrophages) as a guide, performed every 3 to 4 sections. Perilesional NAWM was taken as far as possible from the MS lesions in the same tissue block (at 1–2 cm). Material was collected in prechilled tubes and was stored at −80°C until used. In all the samples, only the WM part of the lesion was dissected. Scale bar = 5 mm.

TABLE 2. List of Primary Antibodies Used for Immunohistochemistry

Primary Antibody	Target	Dilution	Source (reference number)
Anti-SRD5A1 (rabbit)	Amino acids 232 to 256 of 5α- reductase type 1 isozyme	1:500	Prof D.W. Russell, University of Texas Southwestern Medical Center, Dallas, TX (20)
Anti-AKR1C (rabbit)	Rat AKR1C type 9 full protein, which corresponds to human AKR1C type 1–3	1:1000	Prof T. Penning, University of Pennsylvania School of Medicine, Philadelphia, PA (21, 22)
Anti-aromatase (rabbit)	20–Amino acid peptide corresponding to the *N*-terminus of human aromatase	1: 1000	Our laboratory (23)
Anti-HSD3B (rabbit)	Recombinant human HSD3B type 1–2	1:500	Prof J.I. Mason, University of Edinburgh, Edinburgh, UK (24)
Anti-AR (rabbit)	1–20 amino acids of the human androgen receptor	1:2000	Our laboratory (25)
Anti-ERα (rabbit)	Peptide mapping at the C-terminus of ERα of mouse origin	1:200	MC-20; Santa Cruz Biotechnology, Santa Cruz, CA
Anti-ERβ (rabbit)	Amino acids 1–150 of human ERβ	1:10	H-150 sc-8974; Santa Cruz Biotechnology
Anti-PGR (rabbit)	Synthetic peptide corresponding to the C-terminus of human PGR	1:200	PA1-38491; Thermo-Scientific, Rockford, IL
Anti-PGR (mouse)	Recombinant full-length A-form of human progesterone receptor	1:200	M3569 PgR 636; DAKO, Glostrup, Denmark
Anti-GFAP (mouse)	Human glial fibrillary acidic protein (astrocyte marker)	1:200	G3893; Sigma, St Louis, MO
Anti-HLA (mouse)	Human *leukocyte antigen*-DP-DQ-DR (microglia marker)	1:200	M0775; DAKO
Anti-SOX10 (goat)	Sry-related HMG-BOX gene 10 (oligodendrocyte marker)	1:100	AF2864; R&D Systems, UK
Anti-PLP	Myelin proteolipid protein	1:3000	MCA839G; AbD Serotec, Oxford, UK

AKR1C3, aldoketoreductase C3; ERα, estrogen receptor-α; ERβ, estrogen receptor-β; HSD3B, 3β-hydroxysteroid-dehydrogenase.

categories as follows: −, no staining (absent); +, not widespread and transparent staining (weak); ++, widespread staining with individual granules of the reaction product distinguishable (moderate); +++, widespread homogeneous staining (strong); and ++++, widespread and intense opaque staining (very strong; not seen in any of the staining) (see Figure, Supplemental Digital Content 3, http://links.lww.com/NEN/A549 for examples). There was agreement between the observers in 90% of the cases. Estimations of each field/region were averaged per gender group to evaluate the overall sex differences (Table 3).

Isolation and Culture of Human Postmortem Primary Glial Cells

Postmortem human microglia and astrocytes were isolated, cultured, and stimulated as previously described (27, 28). See Materials and Methods, Supplemental Digital Content 2, http://links.lww.com/NEN/A548 for details.

Statistical Analysis

The Shapiro-Wilks test was initially performed in GraphPad Prism version 5.01 (GraphPad Software, Inc., La Jolla, CA) and showed that gene expression data were not normally distributed. Further statistical analysis was conducted with SPSS (version 16.0; SPSS Inc., Chicago, IL). Differences between groups were statistically evaluated by the nonparametric Kruskal-Wallis test for multiple independent samples, followed by Benjamini-Hochberg multiple testing correction. The Mann-Whitney U test was subsequently used for 2 independent samples. Correlations between the mRNA expression of inflammatory- and neurosteroid-related genes studied were calculated using the Spearman rank correlation coefficient. For primary culture experiments, a paired Student *t* test was used. Values of $p < 0.05$ were considered significant. Fold changes were calculated using the median gene expression values.

RESULTS

Gene Expression of Hormone Receptors in MS Lesions and NAWM of Female and Male Patients

An initial comparison of gene expression in chronic active and chronic inactive lesions showed no significant differences between the 2 lesion types within female and male MS groups for all the genes studied (Table, Supplemental Digital Content 4, http://links.lww.com/NEN/A550). Therefore, to increase statistical power, results for the lesions of both types were pooled.

In female MS patients, the mRNA expression of androgen receptor (AR), ERα, and progesterone receptor (PGR) was increased in MS lesions compared with that in WM of controls (Fig. 2A–D). Specifically, AR was 3.3-fold ($p = 0.0012$; Fig. 2A), ERα was 3.1-fold ($p = 0.0033$; Fig. 2B), and PGR was 4.2-fold ($p = 0.0007$; Fig. 2D) higher in lesions than in the CTR samples.

In male MS patients, increased expression of AR (1.9-fold, $p = 0.0011$), ERα (3.4-fold, $p = 0.0005$), PGR (1.8-fold, $p = 0.01$), as well as ERβ (2.5-fold, $p = 0.001$)

TABLE 3. Localization and Cellular Colocalization of Hormone Receptors and Neurosteroidogenic Enzymes in Multiple Sclerosis Lesion Rim, Center, and Normal-Appearing White Matter

Molecule	Intensity in Control White Matter	Localization	Intensity Females	Intensity Males	Intracellular Distribution	Colocalization	Figure
ERα	—	Chronic active rim	+++	+++	Cytoplasm	GFAP	5B,Q
		Cytoplasm			HLA	5R	
		Inactive lesion centers	+++	+++	Cytoplasm	GFAP	5C
ERβ	—	Chronic active rim	+++	+++	Cytoplasm		5F
		Inactive lesion centers	+++	+++	Cytoplasm		5G
AR	+	Chronic active rim	++	++	Nuclei	GFAP	5J, S
		Nuclei			HLA	5T	
		Nuclei			SOX10*	5U	
		Inactive lesion centers	++	++	Nuclei	GFAP	5K
PGR	—	Chronic active rim	++	+	Cytoplasm	GFAP	5N
		HLA					
		Inactive lesion centers	++	+	Cytoplasm	GFAP	5O
		Perilesional NAWM	++	+	Nuclei		6U–W
AKR1C	—	Chronic active rim	++	++	Cytoplasm	GFAP	6A, M
		Inactive lesion centers	++	++	Cytoplasm	GFAP	6B
Aromatase	—	Chronic active rim	++	+++	Cytoplasm	GFAP	6D, N
		Inactive lesion centers	++	+++	Cytoplasm	GFAP	6E
HSD3B	—	Chronic active rim	++	+	Cytoplasm	GFAP	6G,O
		Cytoplasm			HLA	6G,P	
		Inactive lesion centers	+++	++	Cytoplasm	GFAP	6H
		Perilesional NAWM	++	—	Cytoplasm		6R–T
SRD5A1	—	Chronic active rim	+++	+++	Cytoplasm	GFAP	6J,Q
		Inactive lesion centers	+++	+++	Cytoplasm	GFAP	6K
		Perilesional NAWM	++	++	Cytoplasm		6X–Z

AKR1C, aldoketoreductase 1C; AR, androgen receptor; ERβ, estrogen receptor-β; GFAP, glial fibrillary acidic protein; HSD3B, 3β-hydroxysteroid dehydrogenase; NAWM, normal-appearing white matter; PGR, progesterone receptor.

(Fig. 2C) was found in MS lesions compared with that in WM of controls.

Comparison with perilesional NAWM showed an increased expression of AR (2.1-fold, p = 0.0009), ERβ (2.3-fold, p = 0.005), and PGR (2.2-fold, p = 0.0014) in MS lesion of male patients (Fig. 2A, C, D, respectively), whereas AR (1.5-fold, p = 0.015) and PGR expression was increased (2.6-fold, p = 0.0004) in NAWM of female patients versus CTR WM (Fig. 2A, D).

Comparison between female and male expression in MS lesions or MS NAWM or CTR NAWM showed higher expression of PGR mRNA in female MS lesions only (1.5-fold, p = 0.036) (Fig. 2D).

Gene Expression of Enzymes Involved in the Neurosteroid Biosynthetic Pathways in MS Lesions and NAWM of Female and Male Patients

A simplified scheme of the neurosteroid synthetic pathways involved in the gene expression changes and a list of the abbreviations used are shown in Figure 3A. In both female and male MS patients, increased RNA levels of enzyme aldoketoreductase C3 (AKR1C3) (2-fold, p = 0.026, in females; 2.5-fold, p = 0.0027, in males) and translocator protein 18 kDa ([TSPO] a steroidogenesis regulator protein) (1.8-fold, p = 0.016, in females; 1.7-fold, p = 0.0099, in males) were found in MS lesions versus CTR (Fig. 3B, H).

In female MS patients only, the mRNA expression of diazepam binding inhibitor ([DBI] a steroidogenesis regulator protein involved in the cholesterol transport into the mitochondrion) was significantly increased (2.2-fold, p = 0.002) in MS lesions (Fig. 3D) and in MS NAWM (1.6-fold, p = 0.005) compared with that in CTR samples. The DBI was increased in MS lesions of males when compared with that in perilesional NAWM (1.2-fold, p = 0.03). In CTR WM, DBI was higher in males than in females (1.5-fold, p = 0.017) (Fig. 3D).

Moreover, in females, the gene expression of 3β-hydroxysteroid-dehydrogenase (HSD3B) was increased 1.5-fold (p = 0.03) in NAWM and 2.9-fold (p = 0.01) in MS lesions versus males (Fig. 3E). Sulfatase (STS) was also increased in MS lesions in females versus males (1.5-fold, p = 0.013) and in both female and male lesions when compared with the respective NAWM (1.5-fold, p = 0.023 and 1.7-fold, p = 0.027, respectively) (Fig. 3G).

In male MS patients, significantly increased expression of the enzyme AKR1C3 was present in MS lesions versus NAWM (2.2-fold, p = 0.01) (Fig. 3B). In male, but not in female, MS patients, the enzyme aromatase (involved in estrogen synthesis) was 3.2-fold increased compared with that in controls (p = 0.0015; Fig. 3C), whereas the enzyme steroid-5α-reductase, α-polypeptide 1 (SRD5A1) was 1.3-fold (p = 0.0006) higher in both MS lesions and perilesional NAWM (1.2-fold, p = 0.025) versus CTR WM (Fig. 3F). No significant

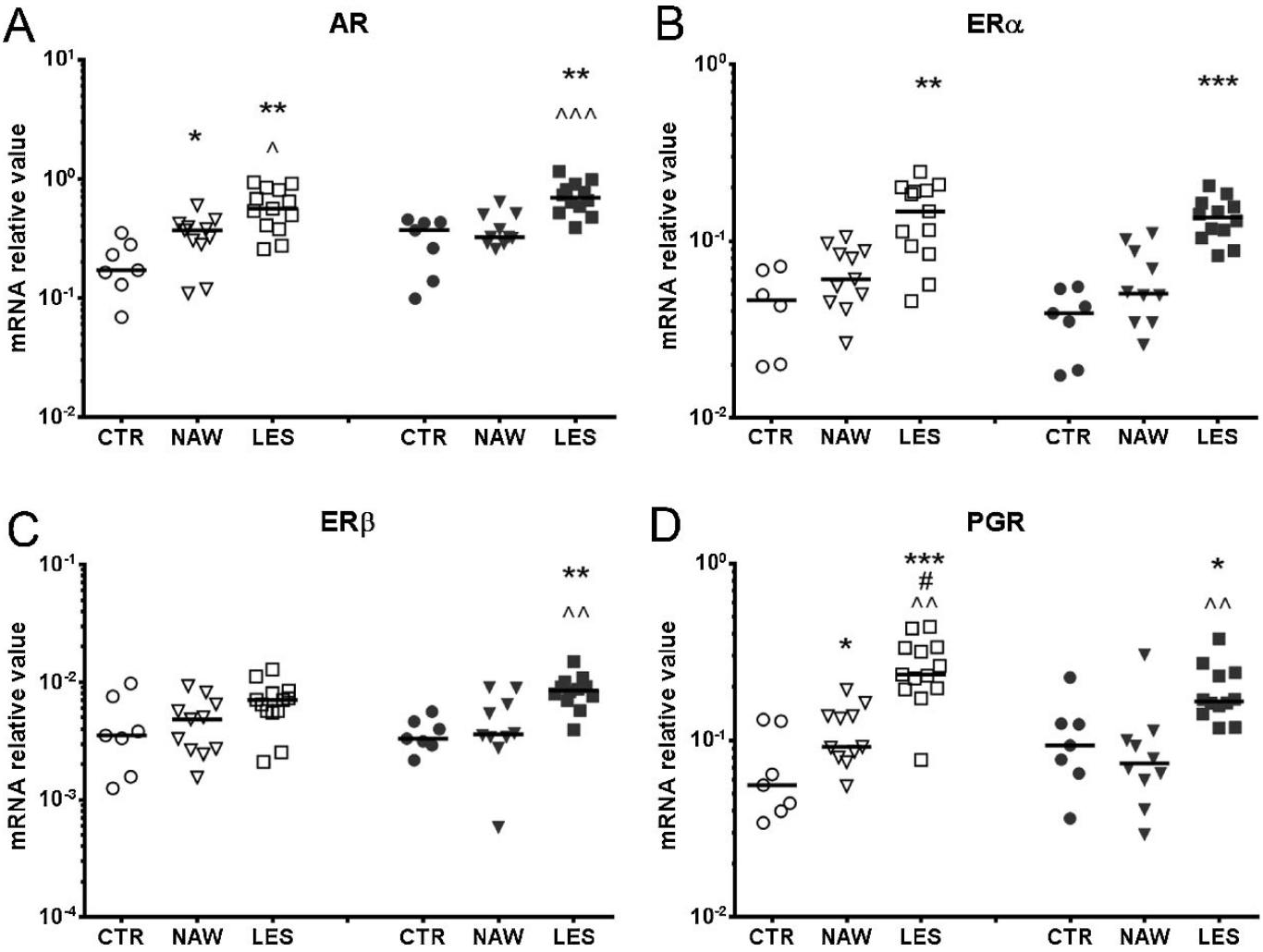

FIGURE 2. Gene expression changes of hormone receptors in female and male multiple sclerosis (MS) patients and controls. **(A–D)** Graphs show comparison of mRNA expression of the genes for androgen receptor (AR) **(A)**, estrogen receptor-α (ERα) **(B)**, ERβ **(C)**, and progesterone receptor (PGR) in control white matter (CTR, n = 7 per gender group), MS normal-appearing white matter ([NAW] females, n = 11; males, n = 10) and MS lesions ([LES] females, n = 13; males, n = 12). Y axis represents the relative value of RNA obtained by the fraction of absolute values with the average of the absolute value of *GAPDH* and *TUB*α housekeeping genes. Scale is expressed in log 10. Circles, triangles, and squares represent control, MS NAW, and MS lesions, respectively. Full or open symbols represent male or female subjects, respectively. *Comparison with control white matter; ^comparison with MS NAW; #comparison between sexes. $*^{\#}$ $p \leq 0.05$; $**^{\wedge\wedge}$ $p \leq 0.01$; $***^{\wedge\wedge\wedge}$ $p \leq 0.001$; Mann-Whitney U test.

changes were found for the other genes examined (Results, Supplemental Digital Content 5, http://links.lww.com/NEN/A551).

Gene Expression of *IL-1*β, *IL-6*, and *TNF* in MS Lesions and NAWM

In MS lesions of males, *IL-1*β gene expression was significantly lower by 5.7-fold (p = 0.0004) versus NAWM. Sex differences were found in WM of controls and MS NAWM, where *IL-1*β levels in males were 8.4-fold (p = 0.022) and 4.6-fold (p = 0.0049) higher than in females, respectively (Fig. 4A). *Interleukin-6* mRNA expression was not significantly different within or between the female and male groups (Fig. 4B). *Tumor necrosis factor* gene expression was increased in MS lesions of male patients versus both CTR WM (2.4-fold, p = 0.01) and female MS lesions (2.8-fold, p = 0.024) (Fig. 4C).

Correlation analysis of gene expression between cytokines and neurosteroid-related compounds shows positive correlations of *TNF* with aromatase (ρ = 0.464, p = 0.01), ERα (ρ = 0.485, p = 0.01), and TSPO (ρ = 0.509, p = 0.02) when data from lesions and perilesional NAWM of females and males were pooled (Fig. 4E, F).

Cellular Localization of Hormone Receptors and Enzymes Involved in Neurosteroid Biosynthesis in MS Lesions and NAWM by Immunohistochemistry

As summarized in Table 3, immunoreactivity of the hormone receptors and neurosteroid synthetic enzymes was

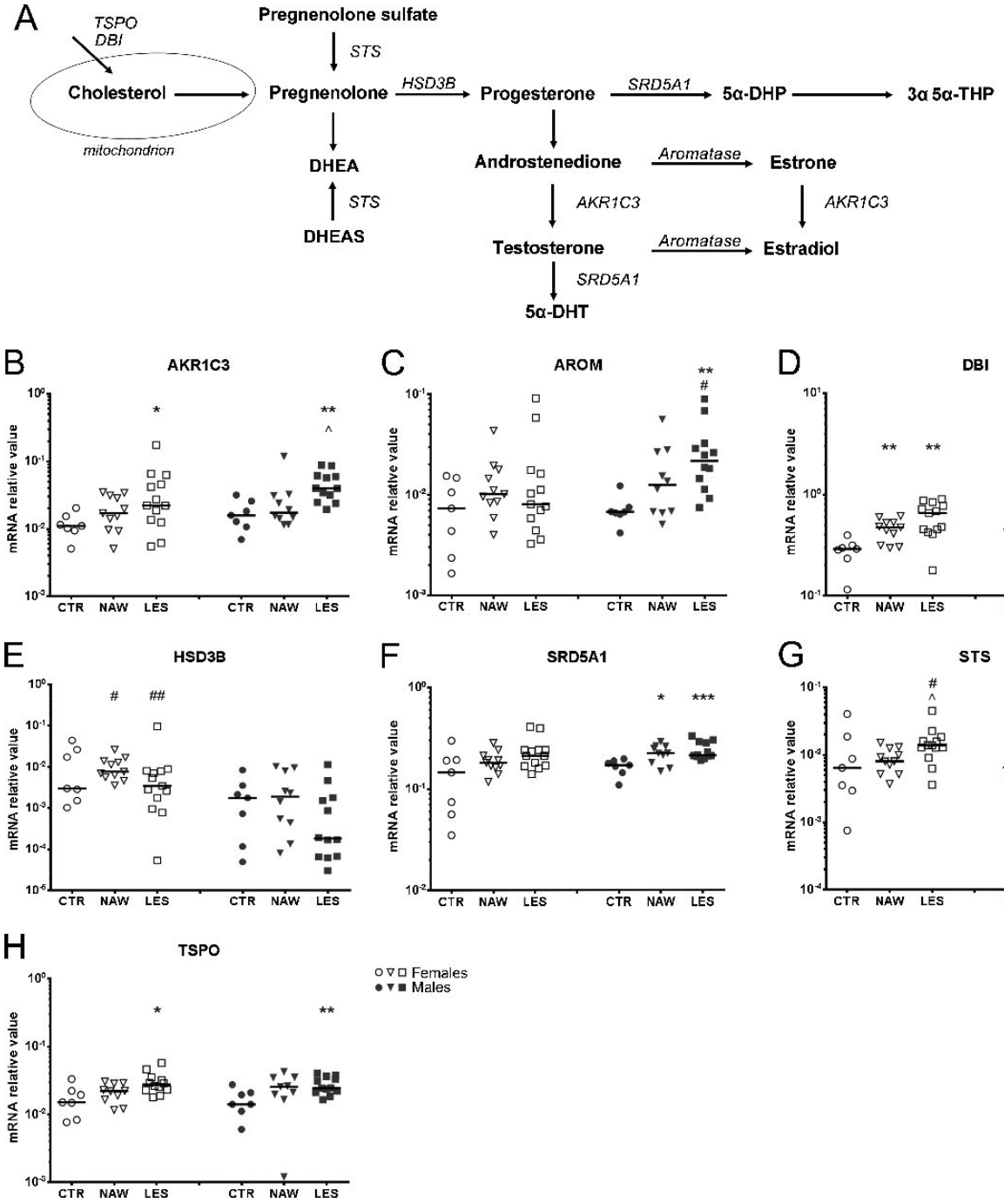

FIGURE 3. Gene expression changes of steroidogenic enzymes and female and male multiple sclerosis (MS) lesions and normal-appearing white matter (NAW). **(A)** Scheme of neurosteroid biosynthetic pathway. **(B–H)** Graphs show comparisons of the mRNA expression of the following genes: AKR1C3 **(B)**, aromatase **(C)**, DBI **(D)**, HSD3B **(E)**, SRD5A1 **(F),** STS **(G)**, and TSPO **(H)** in control white matter (CTR), MS NAW, and MS lesions (LES). AKR1C3, aldoketoreductase C3 (enzyme involved in the synthesis of testosterone and estradiol); DBI, diazepam binding inhibitor (steroidogenesis regulator protein involved in the cholesterol transport into the mitochondrion); DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; HSD3B, 3β hydroxysteroid dehydrogenase (progesterone synthetic enzyme); 5α DHP, 5α dihydroprogesterone; 3 α5 αTHP, 3α5α tetrahydroprogesterone or allopregnanolone; 5α DHT, 5α-dehydrotestosterone; SRD5A1 or 5α reductase type 1 (steroid-5α-reductase, α-polypeptide 1, a rate-limiting enzyme involved in the metabolism of progesterone and testosterone); STS, sulfatase (an enzyme that forms dehydroepiandrosterone or DHEA and pregnenolone from their sulfate esters); TSPO, translocator protein 18 kDa (steroidogenesis regulator protein involved with DBI in the cholesterol transport into the mitochondrion). *Comparison with control NAW; ^comparison with MS NAW; #comparison between sexes. $*^{\wedge\#}$ $p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

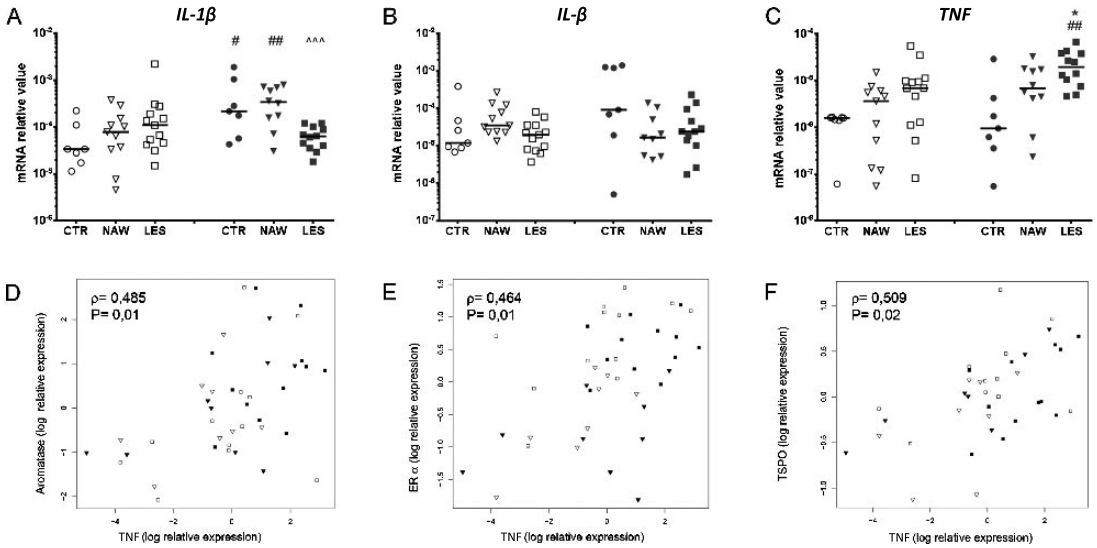

FIGURE 4. Cytokine gene expression changes in female and male multiple sclerosis (MS) lesions (LES) and normal-appearing white matter (NAW) and correlation with steroidogenic enzyme and hormone receptor expression. **(A–C)** Graphs show comparisons of the mRNA expression of the following genes: *IL- 1*β **(A)**, *IL-6* **(B)**, *TNF* **(C)** in control white matter (CTR), MS NAW, and MS LES. *Comparison with control NAW; ^comparison with MS NAW; #comparison between sexes. **(D–F)** Spearman rank correlation of *TNF* gene expression with aromatase **(D)**, ERα **(E)**, and TSPO **(F)** in MS LES and MS NAW from female and male donors. Full or open symbols represent male or female subjects, respectively. Circles, triangles, and squares represent, respectively, CTR NAW, perilesional NAW, and MS LES. TSPO, translocator protein 18 kDa; ERα, estrogen receptor-α. $^{*\#}$ $p \leq 0.05$; $^{\#\#}$ $p \leq 0.01$; $^{\wedge\wedge\wedge}$ $p \leq 0.001$.

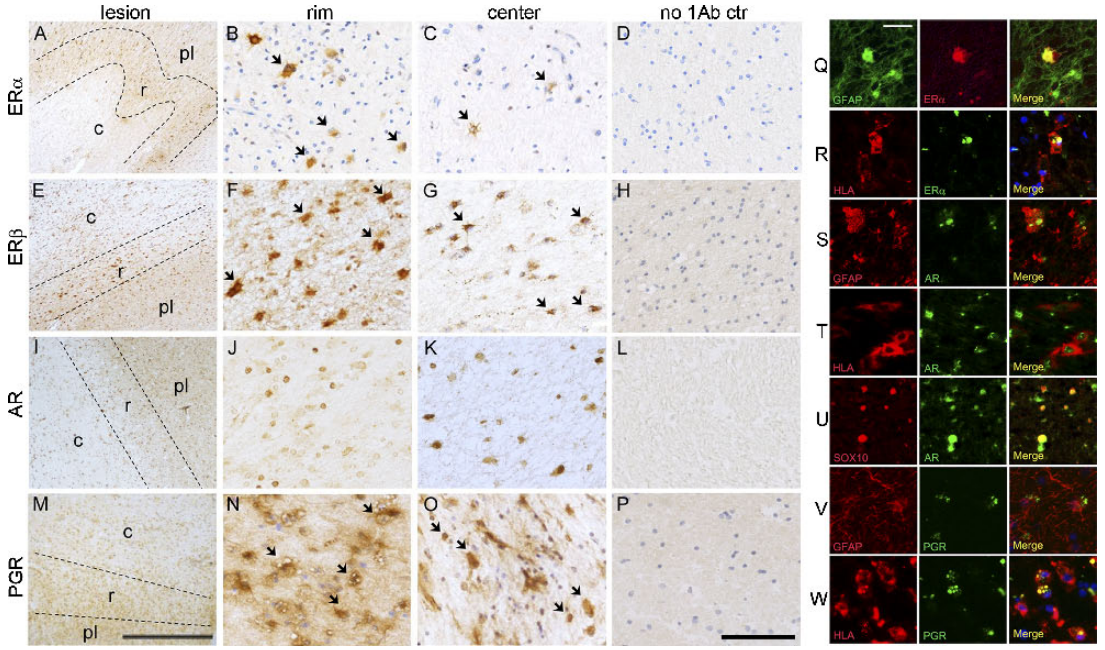

FIGURE 5. Photomicrographs of hormone receptor immunohistochemistry in multiple sclerosis (MS) lesions. **(A–P)** Immunohistochemistry for estrogen receptor-α (ERα) **(A–D)**, ERβ **(E–H)**, androgen receptor (AR) **(I–L)**, progesterone receptor (PGR) **(M–P)**. Panels **(A, E, I, M)** show the overview of the MS lesion with the gliotic core (c), demyelinating rim (r), and perilesional white matter (pl). Scale bar = 500 μm. Panels **(B, F, J, N)** show the lesion rim; **(C, G, K, O)** show the gliotic center; **(D, H, L, P)** show controls (ctr) without primary antibody. Arrows indicate astrocytes where expression is predominantly found. Scale bar = 50 μm. **(Q–W)** Expression of hormone receptors in the rim of MS lesions. Photomicrographs of immunofluorescent double staining for glial fibrillary acidic protein (GFAP) and ERα **(Q)**, HLA and ERα **(R)**, GFAP and AR **(S)**, HLA and AR **(T)**, SOX10 and AR **(U)**, GFAP and PGR **(V)**, and HLA and PGR **(W)** colocalization. Figures show that ERα, AR, and PGR were expressed mostly in the cytoplasm of both GFAP-positive astrocytes **(Q, S**, and **V**, respectively**)** and HLA-positive microglia **(R, T, W**, respectively**)**. In a patient with a lesion close to the subventricular zone, AR was expressed in the nuclei of SOX10-positive oligodendrocytes (U). Scale bar = 25 μm.

mainly localized in the cytoplasm of cells in the chronic active rim and to a lesser extent in the perilesional areas and in inactive lesion centers, and it was colocalized mainly with glial fibrillary acidic protein (Figs. 5, 6). Moreover, Table 3 shows the semiquantitative evaluation of the immunostaining for each molecule analyzed per gender group. Sex differences in immunostaining for aromatase, HSD3B, and PGR were clearly observed.

In particular, aromatase expression was higher in males compared with that in female patients (Fig. 6D, E), whereas HSD3B was higher in female MS patients both in the rim and center of chronic active and inactive lesions (Fig. 6G, H). Moreover, whereas HSD3B was expressed in astrocyte-like cells in NAWM of female MS patients (Fig. 6S), it was not detectable in WM of CTR subjects (Fig. 6R) and was only weakly visible in a few cells of male patients (Fig. 6T).

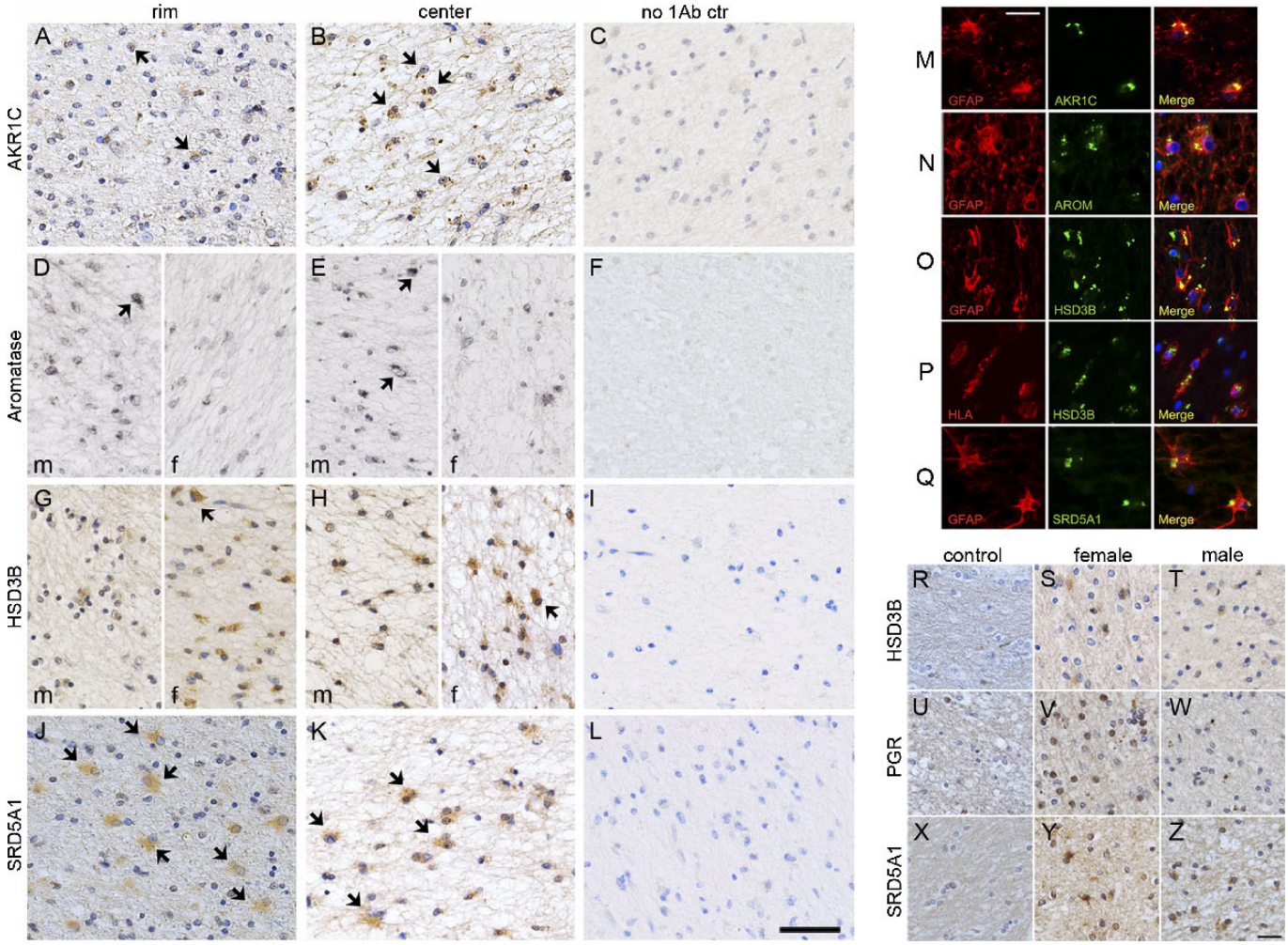

FIGURE 6. Photomicrographs of immunohistochemistry for steroidogenic enzymes and hormone receptors in multiple sclerosis (MS) lesions and normal-appearing white matter (NAWM). **(A–L)** Immunohistochemistry for α-ketoreductase 1C (AKR1C) **(A–C)**, aromatase **(C–F)**, 3β-hydroxysteroid dehydrogenase (HSD3B) **(G–I)**, and 5α-reductase (SRD5A1) **(J–L)**. Panels **(A, D, G, J)** show the lesion rim; **(B, E, H, K)** show the gliotic center. f, female; m, male. Panels **(C, F, I, L)** show staining with no primary antibody added. In male patients, aromatase expression was greater both in the rim **(D)** and in the center **(E)** of MS lesions versus that in females. In female patients, a higher expression of HSD3B was seen in the rim **(G)** and in the center **(H)** of MS lesions versus that in males. AKR1C **(A, B)** and SRD5A1 **(J–K)** were expressed in similar levels in the rim and in the center of MS lesions. All the enzymes were mostly expressed in astrocytes-like cells (arrows). Sections were counterstained with hematoxylin (blue). Scale bar = 50 μm. **(M–Q)** Expression of steroidogenic enzyme in the rim of MS lesion. Photomicrographs of immunofluorescent staining for glial fibrillary acidic protein (GFAP) and AKR1C **(M)**, GFAP and aromatase **(N)**, GFAP and HSD3B **(O)**, HLA and HSD3B **(P)**, and GFAP and SRD5A1 **(Q)** colocalization. AKR1C, aromatase, HSD3B, and SRD5A1 colocalized with GFAP-positive astrocytes, whereas HSD3B was present in HLA-positive microglia. Scale bar = 25 μm. **(R–Z)** Figures show immunohistochemistry for HSD3B **(R–T)**, progesterone receptor (PGR) **(U–W)**, and SRD5A1 **(X–Z)** in control female white matter **(R, U, X)**, NAWM of a female MS patient **(S, V, Y)**, and NAWM of a male MS patient **(T, W, Z)**. Greater expression of HSD3B and PGR is seen in female MS NAWM **(V, Y)**, whereas greater SRD5A1 was seen in male MS NAWM **(W)** compared with that in control white matter **(R, U,** and **X**, respectively**)**. Scale bar = 50 μm.

Nuclear expression of PGR was evident in oligodendrocyte-like cells in the NAWM of female MS patients (Fig. 6V) but was expressed only sporadically in glial cells in WM of female CTR subjects (Fig. 6U). It was not detectable in glial cells of male MS patients (Fig. 6W). Finally, SRD5A1 was not expressed in CTR WM (Fig. 6X) but was seen in astrocyte-like cells, whereas no significant differences were observed in staining intensity comparing female and male NAWM (Fig. 6Y, Z). Detailed semiquantitative evaluation of aromatase, HSD3B, and PGR stainings is shown in Table, Supplemental Digital Content 6, http://links.lww.com/NEN/A552; Table, Supplemental Digital Content 7, http://links.lww.com/NEN/A553; and Table, Supplemental Digital Content 8, http://links.lww.com/NEN/A554. No clear differences were observed between female and male MS lesions by immunohistochemistry for AR, ERα, ERβ, AKR1C3, or SRD5A1.

TNF Effects on Neurosteroid-Related Gene Expression in Human Postmortem Primary Glial Cell Culture

Human primary astrocytes showed increased mRNA expression of ERα when treated with TNF only for 48 hours compared with CTR vehicle-treated cells ($p = 0.011$; Fig. 7A). Human primary microglia showed increased mRNA expression of ERα when treated with TNF plus interferon-γ for 48 hours ($p = 0.03$; Fig. 7B). No significant differences were found in aromatase or in AKR1C3, HSD3B, SRD5A1, PGR, TSPO, DBI, ERβ, and AR gene expression in either glial cell cultures in these experimental conditions.

DISCUSSION

In the present study, we show for the first time differences in expression of enzymes of the sex steroidogenic pathway and sex steroid receptors in MS lesions and NAWM between female and male patients. In male MS lesions, estrogen synthesis and ERβ-mediated signaling are induced; whereas in female MS lesions, progestogen synthesis and signaling are induced. These data indicate that, in response to MS pathology, male MS patients predominantly activate estrogen pathways, whereas females predominantly activate progesterone pathways (Fig. 8).

In male patients, the predominant changes were the local induction of estrogen synthesis and signaling in MS plaques. Expression of aromatase, a key enzyme in the synthesis of estrogens, was increased in male MS lesions compared with that in females. Increased expression of AKR1C3 found in MS lesions may induce the synthesis of both estradiol and testosterone from their less active precursors (29, 30), and aromatase also transforms testosterone into estradiol. Therefore, there is a shift toward the local synthesis of estradiol in MS lesions in men.

Estrogen is clearly beneficial in animal models of MS (10, 11). Estrogen treatment reduced proinflammatory signals (31, 32) and increased anti-inflammatory signals in the spinal cord of mice with experimental autoimmune encephalomyelitis (EAE) (33); it was equally beneficial in males and females (34, 35).

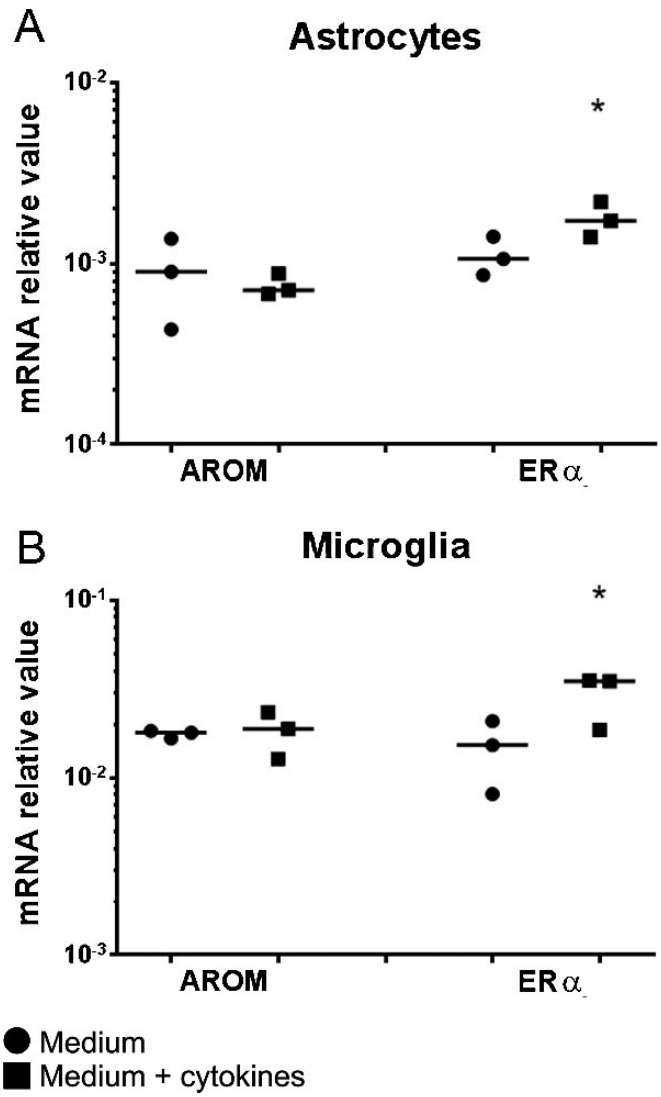

FIGURE 7. Gene expression of aromatase (AROM) and estrogen receptor-α (ERα) in glial cells after tumor necrosis factor (TNF) stimulation. Graphs show mRNA levels of aromatase and ERα in human primary astrocytes from a female donor stimulated in vitro with TNF **(A)** and microglia derived from 3 female donors stimulated with TNF plus interferon-γ (IFN-γ) **(B)** for 48 hours. Increased mRNA expression of ERα was induced in both astrocytes and microglia by this cytokine treatment. * $p \leq 0.05$.

Moreover, in the present study, mRNA expression of ERβ was increased in MS lesions only in male patients. Estrogen receptor-β signaling promotes endogenous neuroprotective and promyelinating mechanisms (36, 37). Notably, the androgen 5-androsten-3β,17β-diol (ADIOL), which functions as a selective modulator of ERβ, prevented EAE in mice and suppressed inflammatory responses of microglia (38). These data suggest that estrogen synthesis and signaling by ERs, particularly ERβ, may represent a compensatory mechanism in male patients to counteract the neuroinflammatory and neurodegenerative process in MS lesions.

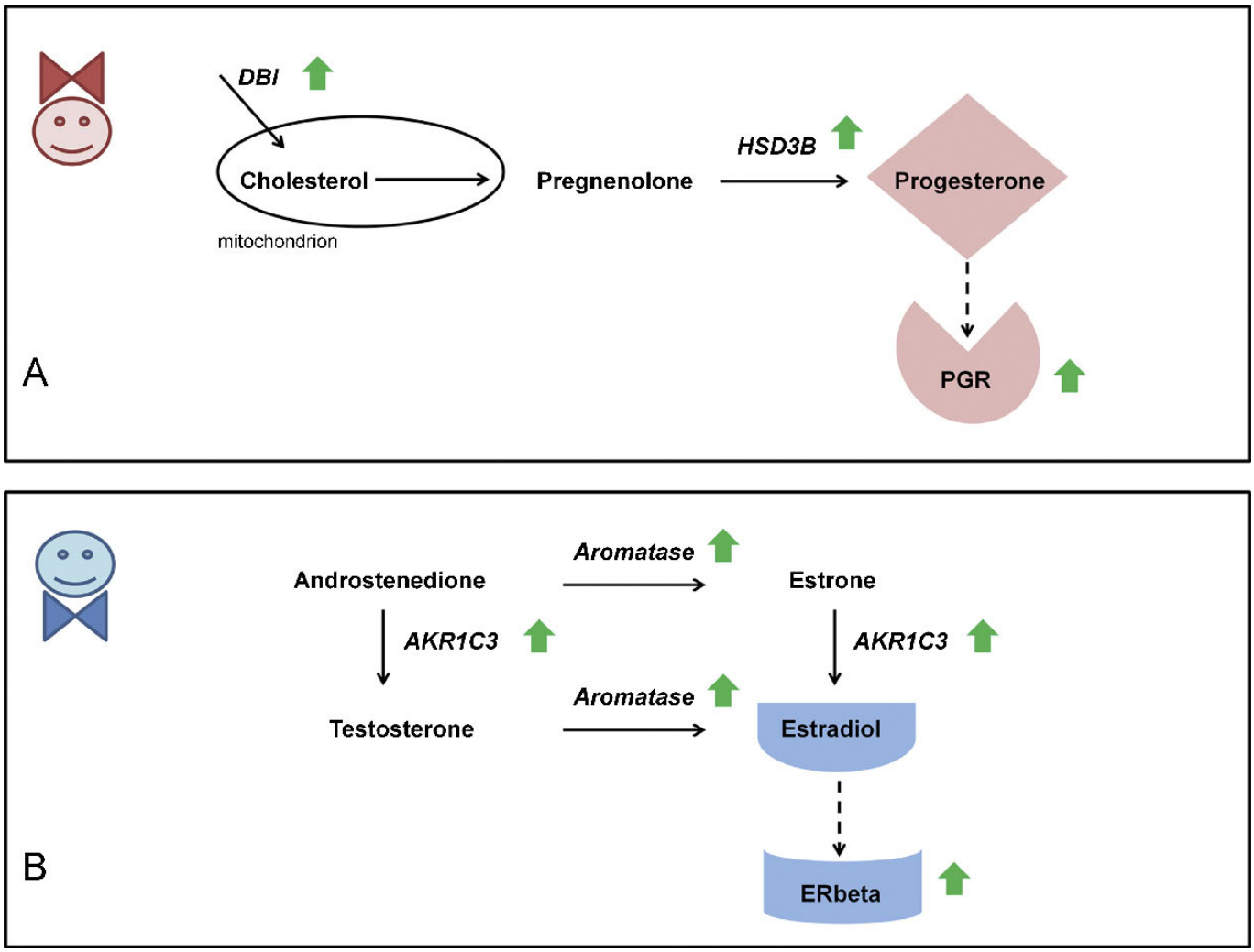

FIGURE 8. Schematic representation of neurosteroid pathway changes in female and male multiple sclerosis (MS) patients. **(A)** In female MS lesion or normal-appearing white matter, increased expression (arrows) of 3β-hydroxysteroid dehydrogenase (HSD3B) enzyme and progesterone receptor (PGR) leads to increased progesterone synthesis and signaling. **(B)** In male MS lesions, increased expression of aromatase and aldoketoreductase C3 (AKR1C3) enzymes leads to estradiol synthesis, whereas increased estrogen receptor β (ERβ) leads to increased estrogen signaling.

In female MS lesions, the most significant changes were the increased gene expression of PGR and higher levels of progesterone synthetic enzyme HSD3B versus males. Indeed, higher progesterone synthesis was seen in the spinal cord of female rats compared with that of males in chronic EAE (39). Functionally, progesterone and its metabolite DHP have been shown to ameliorate the clinical signs of EAE (40), reduce reactive gliosis, promote remyelination and oligodendrocyte differentiation (41, 42), and decrease inflammatory activity of microglia (43). These data suggest a higher capacity for females to increase progesterone synthesis and signaling that might represent an additional protective mechanism in the female CNS during MS and might be partially responsible for the more benign course of the disease as compared with that in males (2).

Although it was not possible to distinguish a clear sex difference in ERβ by immunohistochemistry, it was possible in the present study to identify greater immunostaining intensity for PGR and HSD3B in female MS lesions and in aromatase in lesion center and rim in male MS patients. In our opinion, this supports the possibility that the balance between estrogen and progestogen synthesis may differ in female and male MS lesions. In future studies, we plan to detect the levels of progestogens and estrogens directly in MS lesions of female and male patients to confirm these initial observations.

Androgen receptor mRNA was increased in both male and female MS lesions. From rodent studies, there is some evidence that androgens acting through the AR reduce neuroinflammatory and neurodegenerative processes and promote remyelination (44, 45). Androgen signaling may, therefore, have an important role in neuroinflammatory disease; our findings indicate that alterations in androgen signaling also take place in MS.

Two of our findings are in contrast with the studies of Noorbakhsh et al (17) who found reduced mRNA of the allopregnanolone synthetic enzymes SRD5A1 and AKR1C2 in NAWM of MS patients. We found that SRD5A1 was increased in males and AKR1C2 was unchanged in the NAWM of both sexes. Confounding factors such as the absence of matching for postmortem parameters of patient cohorts, long postmortem delay (>15 hours), and therapies likely to interfere with steroidogenesis (e.g. finasteride, corticosteroids, NSAIDs) in their study might account for the differences. Another possible explanation may be a difference in the exact location of the tissue samples. In our study, the NAWM was dissected perilesionally 1 to 2 cm from the lesion rim, and we have shown that, in NAWM of male patients, reactive astrocytes are present and are a source of the increased expression of SRD5A1 we found, whereas no specific information on dissection or immunocytochemistry localization was provided by Noorbakhsh et al (17). We found higher TNF in male MS lesions compared with that in females, which might be related to a sexually dimorphic response to an inflammatory stimulus (46).

Tumor necrosis factor expression was positively correlated with the increases in aromatase and ERα. Furthermore, TNF treatment upregulated ERα gene expression in cultured human astrocytes and microglia, corroborating the idea that it contributes to induce ERα expression in MS lesions. We show that neurosteroidogenesis takes place mainly in reactive astrocytes in the rim and center of MS lesions. Many studies have observed that neurosteroid synthesis by reactive astrocytes is part of the protective and anti-inflammatory response to traumatic injury (10, 47). Moreover, induction of hormone receptor expression in astrocytes suggests that they are the key cells responsible for mediating protective actions of neurosteroid signaling in MS lesions. Notably, anti-inflammatory effects of steroids mediated by astrocytes have been found previously in EAE (48, 49), where the neuroprotective and anti-inflammatory effects of ERα ligands were abolished in astrocytic ERα–knockout mice but not neuronal ERα knockouts (50).

In addition, some microglia in the lesion rim express AR, ERα and PGR, showing that they are also neurosteroid-responsive. In mice, estrogen and progesterone both inhibit microglial activation (15) in response to lipopolysaccharide (43, 51, 52). Our data, combined with these findings, suggest that progesterone and estrogens synthesized by astrocytes in MS have the potential to reduce inflammatory activities in microglia by signaling through PGR or ERα.

Progesterone and estrogen synthesis and signaling might, therefore, represent an endogenous coping mechanism to fight the inflammatory, demyelinating, and degenerative processes occurring in MS. We postulate that this mechanism is only partially effective and, therefore, exogenous administration of steroids could very well additionally ameliorate the disease course. Specifically, because we have shown that expression of ERβ is significantly increased in MS lesions of males compared with that of females, the signaling of this receptor may represent a therapeutic target for male patients. A strategy based on ERβ ligands (e.g. ADIOL), alone or in combination with the current immunosuppressive therapy (37), might increase neuroprotection and counteract the progression of the disease in male patients. Our data also suggest that progesterone administration might be beneficial in both sexes because progesterone synthetic enzyme and receptor expression is increased in both male and female MS lesions and in female NAWM.

In summary, we have shown for the first time that the expression of sex steroidogenic enzymes and hormone receptors is increased in MS lesions and NAWM in a gender-specific manner. Our findings indicate that, in males and females, the balance between local production of progesterone and estrogen differs, which may account for sex differences in lesion development. In addition, TNF might have an important role in inducing estrogen synthesis and signaling to cope with neuroinflammatory and degenerative stimuli, particularly in male patients. Finally, increased progestogen synthesis and PGR expression in NAWM of female patients indicate that changes in neurosteroid pathway occur before lesion development and may represent an endogenous protective mechanism. The gender-specific changes in steroidogenesis and signaling we describe might be important in the design of sexual dimorphic future therapeutic strategies.

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