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Estradiol Is Required for a Proper Immune Response to Bacterial and Viral Pathogens in the Female Brain

Geneviève Soucy,* Guy Boivin,† Fernand Labrie,* and Serge Rivest2*

Although the neuroprotective effects of estrogens are well recognized, the exact mechanisms involved in the ability of these sex steroids to protect the cerebral tissue still remain unclear. We tested in our study the hypothesis that estradiol (E2) modulates the innate immune response and expression of genes encoding proteins that provide survival signal to neurons during infection. Mice received a single systemic or cerebral injection of LPS to trigger a robust but transient inflammatory reaction in the brain. The endotoxin increased transcriptional activation of genes encoding TLR2, TNF-α, and IL-12 in microglial cells. Expression of these transcripts was largely inhibited in the brain of ovariectomized mice at time 24 h postchallenge. E2 replacement therapy totally rescued the ability of the endotoxin to trigger microglial cells and these permissive effects of E2 are mediated via the estrogen receptor (ER)α. Indeed, ERα-deficient mice exhibited an inappropriate reaction to LPS when compared with ERβ-deficient and wild-type mice. This defective innate immune response was also associated with a widespread viral replication and neurodegeneration in ovariectomized mice inoculated intranasally with HSV-2. These data provide evidence that interaction of E2 with their nuclear ERα plays a critical role in the control of cytokines involved in the transfer from the innate to adaptive immunity. This transfer is deviant in mice lacking E2, which allows pathogens to hide from immune surveillance and exacerbates neuronal damages during viral encephalitis. The Journal of Immunology, 2005, 174: 6391–6398.

An infected host recognizes invading microorganisms via elements called pathogen-associated molecular patterns (1). Interaction between pathogen-associated molecular patterns with their cognate receptors expressed on the surface of specific cells of the immune system leads to an innate immune response that is characterized by transcriptional activation genes encoding cytokines, chemokines, and many other proteins involved in the acute-phase response. In this regard, Gram-negative cell wall component LPS is a useful tool to trigger the events that take place during the innate immune and inflammatory response (2). The endotoxin injected either systemically or centrally also triggers proinflammatory signaling and gene expression in microglial cells throughout the CNS (3–8). The main role of this rapid inflammatory response is to eliminate the pathogen and prepare the transfer from the innate to adaptive immunity.

This innate immune response is under the control of numerous factors including glucocorticoids that are the most potent endogenous inhibitory feedback on proinflammatory signal transduction pathways (9). Although more subtle, sex steroids have been shown to modulate the immune system. Estrogen replacement therapy decreased the number of circulating monocytes in postmenopausal women (10) and estrogen treatment increased Fas ligand expression in monocytes (11). Anti-inflammatory properties have also been attributed to these steroids (12–15) and such effects on the immune system have been suggested to contribute to the neuroprotective roles of estrogens (16–18). However, the exact mechanisms involved in the ability of estrogens to modulate the innate immune response in the brain still remain poorly understood.

Estradiol (E2) is the main secreted estrogen. It acts via two nuclear receptors, namely estrogen receptor (ER)α and ERβ. These two receptors are highly homologous and they share many functional characteristics, but their transcriptional activities are different and complementary (19). They are both expressed in the brain, but the anatomic distribution and subcellular colocalization are highly different between the receptor subtypes (20). The present series of experiments tested the hypothesis that E2 plays a critical role in the control of the innate immunity in the female brain. We subsequently determined the receptor involved in these events in ERα-deficient (ERKO) and ERβ-deficient (BERKO) mice. Finally, a mouse model of viral encephalitis (21) was used to investigate the role of E2 in the mechanisms that control the transfer from innate to adaptive immune reactions.

We provide in our study compelling evidence that E2 is required for the rapid production of key cytokines involved in the transfer from the innate to adaptive immunity. Alteration of this positive feedback by these sex steroids on microglial cells has direct consequences on the cerebral tissue.

Materials and Methods

Animals

Adult intact and ovariectomized (OVX) CD1 mice (~25 g of body weight; Charles River Breeding Laboratories) and C57BL/6j mice (~35–45 g of body weight) were acclimated to standard laboratory conditions (14 h light, 10 h dark cycle; lights on at 0600 h and off at 2000 h) with free access to mouse chow and water. Knockout mice were provided by Prof. P. Cham- bon (Collège de France, Strasbourg, France) (22). Appropriate housing

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conditions (microisolators) and surveillance procedures were used to maintain a disease-free state within the colony. Mice heterozygous for the mutated Erα gene were bred and the first generation offspring were genotyped by PCR analysis of tail genomic DNA. For ERKO mice, the following PCR primers were used: P1, 5′-TGCGCGATAAATAAGACATC-3′; P2, 5′-ATGCTCTTCCCTGACAC-3′; P3, 5′-GGCTTACCCACCTTCTCTGAGGAC-3′ and P4, 5′-TGGCCTTTGCAAGACCTTCATAT-3′. The sizes of the P1-P2 and P3-P4 PCR fragments in wild-type mice are 387 and 415 bp, respectively. The size of the P3-P4 fragment amplified from the ERKO allele is 255 bp. Tail DNA genotyping of BERKO mice was performed using the primers: P1, 5′-AAGTGTAAGCCAGCCCAAGG-3′; P2, 5′-AGCTCCCTGTAGCTCATAT-3′; and P3, 5′-AAGGGCATGCTCCAGAATGC-3′, which yield ∼380 bp fragments for wild-type mice (++) and 240 bp fragments for homozygous animals (−−) (P1 and P3 primers), and both fragments for heterozygotes (+−). Animal breeding and experiments were conducted according to Canadian Council on Animal Care guidelines, as administered by the Laval University Animal Care Committee. A total of 150 mice were assigned to four protocols that contained different treatments and postinjection times (3, 9, and 24 h and 3, 5, 7, 8, and 10 days).

Surgeries and treatments

Intact and OVX mice receiving intracerebral injections of LPS (2.5 μg/μl from Escherichia coli, serotype O55:B5, product no. L-2880; Sigma-Aldrich) or sterile saline solution (0.3%) were anesthetized with an i.p. injection (10 ml/kg body weight) of a mixture of ketamine hydrochloride (15 mg/ml) and xylazine (1 mg/ml) and placed in a stereotaxic apparatus (David Kopf Instruments). The striatum was then reached (−2.0 mm lateral and −3.0 mm dorsal to the bregma) with a 33-gauge stainless steel cannula (Plastics One) that was connected to a 50-μl Hamilton syringe with an intramedic polyethylene tubing (PE-50; Clay Adams). A volume of 1 μl was infused over 2 min by means of a microinjection pump (model A-99; Raziel Scientific Instruments). These coordinates were selected on the basis of previous data showing a robust hybridization signal and reliable pattern of cytokine gene expression over the ipsilateral cerebral cortex, hippocampus, corpus callosum, and basal ganglia following a single bolus of LPS (5). These coordinates were therefore used to represent a general mechanism of immune response in the cerebral tissue. At different times after the injection (24 h, and 3 and 7 days), mice were deeply anesthetized via an i.p. injection (100 μl) of a mixture of ketamine hydrochloride (91 mg/ml) and xylazine (91 mg/ml) and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borax buffer (pH 9.5 at 4°C). A second protocol was developed to verify the ability of Erα to modulate the inflammatory reaction by microglial cells in response to systemically injected LPS. One group of OVX mice was anesthetized with isoflurane and were implanted s.c. with a silastic implant of E2 (cholesterol to 17α-estradiol; David Kopf Instruments). The striatum was then reached (−2.0 mm lateral and −3.0 mm dorsal to the bregma) with a 33-gauge stainless steel cannula (Plastics One) that was connected to a 50-μl Hamilton syringe with an intramedic polyethylene tubing (PE-50; Clay Adams). A volume of 1 μl was infused over 2 min by means of a microinjection pump (model A-99; Raziel Scientific Instruments). These coordinates were selected on the basis of previous data showing a robust hybridization signal and reliable pattern of cytokine gene expression over the ipsilateral cerebral cortex, hippocampus, corpus callosum, and basal ganglia following a single bolus of LPS (5). These coordinates were therefore used to represent a general mechanism of immune response in the cerebral tissue. At different times after the injection (24 h, and 3 and 7 days), mice were deeply anesthetized via an i.p. injection (100 μl) of a mixture of ketamine hydrochloride (91 mg/ml) and xylazine (91 mg/ml) and then rapidly perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M borax buffer (pH 9.5 at 4°C).

Finally, intranasal inoculation of HSV-2 was used as a model of viral encephalitis, which causes strong innate and adaptive immune responses in the brain (21). Intact and OVX CD1 mice were anesthetized for a few seconds by inhalation of isoflurane (Janssen-Ortho). A total of 20 μl of MEM (Invitrogen Life Technologies) containing either no virus (vehicle) or 104 PFU of HSV-2 strain was inoculated intranasally in the animals. The viral strain was plaque-purified three times from the original isolate of an HSV-infected subject. Mice were deeply anesthetized and perfused at different days after the HSV-2 inoculation (3, 5, 7, 8, and 10 days).

Brain preparation and histology

Rapidly after the perfusions, brains were removed from the skull, postfixed for 1–3 days, and then placed in 10% sucrose diluted in the solution of 4% paraformaldehyde-borax buffer overnight at 4°C. The frozen brains were mounted on a microtome (Reichert-Jung; Cambridge Instruments) and cut into 25-μm coronal sections from the olfactory bulb to the end of the medulla. The sections were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, 30% ethylene glycol, 20% glycerol) and stored at −20°C. Plasmids were linearized and sense and antisense riboprobes were synthesized as described in Table 1. Hybridization histochemical localization of the different transcripts and their colocalization within microglial cells was conducted on every twelfth section of the whole rostrocaudal extent of each brain using 15S-labeled cRNA probes as previously described (23, 24).

Table 1. Plasmids and enzymes used for the synthesis of the cRNA probes

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Vector</th>
<th>Insert</th>
<th>Antisense Probe</th>
<th>Sense Probe</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse IL-12p40</td>
<td>pCL-Neo</td>
<td>1050 bp</td>
<td>XhoIT3</td>
<td>NorIT7</td>
<td>Dr. K. Pahan (University of Nebraska Medical Center, Lincoln, NE)</td>
</tr>
<tr>
<td>Mouse IFN</td>
<td>pGEMEX</td>
<td>550 bp</td>
<td>HindIII/T3</td>
<td>EcoRI/Sp6</td>
<td>Dr. I. Campbell (Scripps Research Institute, La Jolla, CA)</td>
</tr>
<tr>
<td>Mouse TLR2</td>
<td>PCR blunt II</td>
<td>2,278 kb</td>
<td>EcoRV/Sp6</td>
<td>SpeI/77</td>
<td>PCR amplification provided by Dr. D. Radzioch (McGill University Montréal, Quebec, Canada)</td>
</tr>
<tr>
<td>Mouse TNF</td>
<td>Bluescript K II*</td>
<td>1300 bp</td>
<td>PstI/T3</td>
<td>BamHI/T7</td>
<td>Subcloned from a PUc19 plasmid provided by Dr. D. Radzioch (McGill University Montréal, Quebec, Canada)</td>
</tr>
</tbody>
</table>

*The DNA fragment of 2.278 kb corresponding to the almost complete coding sequence (2.355 kb) of the reported mouse TLR2 mRNA (nucleotide 307–2661; GenBank accession no. AF185284) was amplified by PCR from a cDNA macrophage B10R cell line library using a pair of 23 bp oligonucleotide primers complementary to nucleotides 323–345 (5′-GGCGCTTCTGTAGCTTGGCC-3′) and to 2579–2601 (5′-GGGCCACTCCAGTTAGTCTTGGG-3′).
The single LPS treatment (Fig. 2, top row). This result contrasts with the robust and widespread signal for the proinflammatory cytokine transcript in the ipsilateral side 24 h and 3 days after a single infusion with the bacterial cell wall component (2.5 μg of LPS/μl, second row). B, Dual-labeling combining immunocytochemistry with in situ hybridization to identify the phenotype of IL-12-, TNF-α, and TLR2-expressing cells. Microglia were labeled by immunohistochemistry using an Ab directed against iba1, brown ramified cells). The different transcripts were thereafter hybridized separately on adjacent coronal sections via a radioactive in situ hybridization technique (silver grains). Black arrows indicate double-labeled cells (agglomeration of silver grains within brown ramified cells). All (100%) IL-12-, TNF-α, and TLR2-expressing cells were localized with the iba1-immunoreactive signal, but not all microglia contained positive messages for the immune genes.

mRNA (Fig. 1A, top row). This localized pattern of positive TNF-α-expressing cells lining the cannula track contrasts with the intense and widespread transcriptional activation of TNF-α across the hemisphere of the ipsilateral side. The cerebral cortex, hippocampus, corpus callosum, and basal ganglia exhibited an intense TNF-α mRNA signal 24 h after the single bolus of LPS (Fig. 1, 24 h LPS). The message spread throughout the entire hemisphere at 3 days and largely declined 7 days following the endotoxin administration. The pattern for the mRNA encoding TLR2 was similar, but the signal for IL-12p40 was more localized to the infusion site and vanished 3 days after the intrastriatal LPS infusion (Fig. 2). In this particular case, numerous IL-12p40-expressing cells were found in the cerebral cortex of mice killed 24 h after the single LPS treatment (Fig. 2, top left panels). In contrast, TLR2 mRNA signal remained strong up to 7 days post LPS infusion (Fig. 2B, bottom).

To ascertain the phenotype of IL-12p40-, TNF-α-, and TLR2-expressing cells, immunocytochemistry was combined with in situ hybridization. We used in this study an Ab directed against anti-ionized calcium binding adapter molecule 1 (iba1) to label cells of myeloid lineage (macrophages and microglia) (27, 28). All three transcripts colocalized with iba1-immunoreactive cells providing clear anatomic evidence that microglia are the key cells responsible for the cerebral innate immune response in the presence of a cell wall component derived from Gram-negative bacteria (Fig. 1B).

Role of E2 in the control of cerebral innate immunity
Of interest is the aberrant innate immune reaction in the brain of OVX mice (Fig. 2). Indeed, OVX mice failed to respond to a single bolus of LPS at 24 h; although the signal for IL-12, TNF-α, and TLR2 was robust across the ipsilateral side of intact mice, no positive cells were found in this region of OVX mice after the endotoxin treatment. The number of positive IL-12 cells and the OD values for TNF-α and TLR2 signal were not significantly different for OVX-LPS, OVX-vehicle, and intact-vehicle groups of mice (Fig. 2B, 24 h). The endotoxin was therefore only able to rapidly trigger microglial cells in the brain of intact animals. However, the brains of OVX mice responded to the single bolus of LPS at 3 and 7 days postadministration. TNF-α and TLR2 mRNA signals were similar and significantly higher in the brain of both LPS-treated groups than in vehicle-administered mice 3 days following the intracerebral treatment (Fig. 2B). The gene encoding IL-12p40 still remained undetectable at 3 days post LPS infusion in intact and OVX mice. Therefore, OVX completely abolished the synthesis of this particular cytokine in response to intraparenchymal endotoxin infusion.

We have consequently verified whether these effects were specific to a model of intracerebral endotoxin insult and performed another series of experiments in intact and OVX mice challenged with a systemic bolus of LPS. We have previously shown that this dose of LPS (1 mg/kg) causes a profound increase in the transcription of the gene encoding TLR2 first with the circumventricular organs (CVOs), choroid plexus, leptomeninges, and microcapillaries and thereafter across the brain parenchyma in a migratory-like pattern (24). Fig. 3 depicts representative examples of such a phenomenon in the choroid plexus/adjacent regions and microcapillaries/adjacent areas. A very strong signal was detected in some structures 24 h after the single i.p. LPS bolus. Scattered TLR2-expressing cells were also found in the cortex and in various regions of the CNS (Fig. 3, arrows). Save for the groups of cells
associated with the choroid plexus and CVOs, TLR2 transcript levels remained low to undetectable in parenchymal microglia of OVX animals challenged with the bacterial cell wall component (Fig. 3, middle column). Replacement therapy with E2 completely rescued the ability of LPS to trigger TLR2 gene expression throughout the cerebral tissue (Fig. 3, right column).

These data therefore clearly support the concept that E2 is required for expression of the normal complement of proinflammatory proteins in response to both intracerebral and systemic LPS treatments in the mouse brain. To determine the receptor involved in these effects, we used mutant mice deficient in either ERα (ERKO mice) or ERβ (BERKO mice) that were challenged with a single i.p. LPS bolus. Similar to OVX animals, ERKO mice were less sensitive to the endotoxin and hybridization signal for the gene encoding TLR2 was significantly lower in ERKO mice than their wild-type littermates. In contrast, a strong increase in TLR2 gene expression took place in the CNS of both BERKO and wild-type mice. Semiquantitative analysis in the region of the median eminence/arcuate nucleus indicated that TLR2 mRNA levels were significantly lower in ERKO mice than the other LPS-treated groups (Fig. 4, bottom). Actually, the OD value was four times lower in ERKO mice compared with their wild-type littermates 24 h after the systemic LPS challenge.

Role of E2 in a mouse model of viral infection

The next series of experiments investigated the consequences of an altered innate immune response in a model of viral infection, which involves both innate and adaptive immunities. The HSV-2 is neurotrophic and its replication in the CNS is associated with a robust inflammatory response in the hindbrain of male mice (21). Female mice inoculated with HSV-2 also exhibited a strong hybridization signal for numerous inflammatory genes and the viral thymidine kinase (index of HSV replication) in the pons and medulla 7–10 days postinoculation. However, the signals were low or barely detectable in more rostral regions, such as the hypothalamus, mammillary bodies, and thalamic structures of intact female mice (Fig. 5). In contrast, a widespread pattern of HSV-immunoreactive neurons and TLR2-expressing cells was found across the hypothalamus of OVX mice killed 10 days after being inoculated with HSV-2. This microglial reactivity (TLR2-positive cells) (Fig. 5C) is a direct consequence of an increased neurovirulence (Fig. 5A) and HSV-induced neuronal damage (Fig. 5B). The latter was estimated by immunohistochemistry using a primary Ab directed against the neuronal marker NeuN, which vanishes rapidly in degenerating neurons (29). As depicted in Fig. 5B, HSV-immunoreactive neurons no longer contained nuclear NeuN staining in the

FIGURE 2. The innate immune response is altered in the brain of ovariectomized (OVX) mice. A, Photomicrographs depicting hybridization signal of IL-12p40 (top), TNF-α (middle), and TLR2 (bottom) mRNA 24 h after the intrastral LPS infusion. The coronal sections were taken from the x-ray film (Biomax; Kodak), whereas darkfield photomicrographs were taken from the same section dipped into the nuclear emulsion milk (NTB-2; Kodak). B, Quantitative analysis of the different cytokine transcripts in the ipsilateral side. The number of IL-12-expressing cells (top) in four coronal sections of each animal with OD measurements (middle and bottom) are shown. Values are mean ± SEM of three to four mice per group, and statistical analysis was performed using a two-way ANOVA followed by a Bonferroni-Dunn posthoc test (StatView 4.5). * Significantly different (p < 0.05) from their corresponding vehicle-treated groups. Scale bar represents 500 μm. IL, intact LPS; OL, OVX LPS; IV, intact vehicle; OV, OVX vehicle.
FIGURE 3. Role of 17β-E2 on systemic LPS-induced innate immune response in the brain. Mice received a single i.p. injection of either vehicle (sterile saline) or LPS (1 mg/kg) and were killed 24 h afterward. The coronal sections depict representative patterns of TLR2 hybridization signal in intact, OVX, or OVX mice that received replacement therapy with 17β-E2. Please note the robust expression of TLR2 mRNA in the brain of intact (left column) and OVX+E2 (right column) mice 24 h following the i.p. LPS administration, whereas OVX mice exhibited an attenuated response to the endotoxin (middle column). Coronal sections were taken from x-ray films (Biomax; Kodak), whereas the same sections dipped into nuclear emulsion (NTB-2; Kodak) were used for the darkfield photomicrographs (bottom). SH, sham implant.

FIGURE 4. The permissive influence of 17β-E2 on the CNS innate immunity is dependent on ERα. Both ERα (ERKO, top panels) and ERβ (BERKO, middle panels) groups of mice were killed 24 h following a single i.p. injection of LPS (1 mg/kg). TLR2 gene expression was used as a general index of innate immune response in the brain that was stimulated by the endotoxin and significantly prevented in the brain of ERKO mice (KO+LPS). The semiquantitative analysis was performed in the region depicted by the rectangle illustrated on the coronal section (top left). Values are mean ± SEM and statistical analysis was performed using a two-way ANOVA followed by a Bonferroni-Dunn posthoc test (StatView 4.5). *, Significantly different (p < 0.05) from their corresponding vehicle-treated groups. †, Significantly different (p < 0.05) from all the others LPS-treated groups. Note that vehicle-treated mice exhibited very low TLR2 mRNA signal, which explains the nondetectable OD levels in most of the control groups (■).
hypothalamus of OVX mice. Intact mice inoculated with the virus had very few (if any) positive HSV-immunoreactive neurons in these rostral regions, but numerous HSV+/NeuN− neurons were found across the hindbrain of both intact and OVX mice (data not shown). These data indicate that an inadequate innate immune reaction to HSV-2 is associated with an increased neurovirulence and a more widespread neurodegeneration in the brain of OVX mice.

Discussion
The present study shows that E2 plays an essential role in modulating the proinflammatory signaling and gene expression in microglial cells of mice. An aberrant reaction took place in the brain of OVX mice challenged with LPS either centrally or systemically. These permissive properties of E2 for the inflammatory responses are mediated via ERα, because ERKO and OVX mice had similar altered microglial reactivity to LPS. The neuroanatomical organization of ERα and ERβ across the rodent brain has indicated a widespread distribution of target sites for estrogen to influence multiple cellular functions in a site- and receptor-dependent manner. Indeed, several regions were found to express both receptor subtypes, but fine differences were reported in terms of intensity and positive signal (20). Moreover, the mRNA encoding ERα has a more widespread distribution than the ERβ, and ERα is the only transcript expressed at different levels of all the sensorial CVOs (20). These data are of great interest because these regions are considered to be immune sentinels of the brain during systemic infection.

The innate immune response is driven through specific recognition systems, such as interaction between LPS and its receptors CD14 and TLR4. Both CD14 and TLR4 receptors are constitutively expressed in the CVOs, and circulating LPS is able to cause a rapid transcriptional activation of genes encoding proinflammatory molecules in these organs (6, 8, 30). Constitutive expression of CD14, TLR4, and ERα in CVOs provides direct anatomical evidence that estrogen may enhance the LPS signaling events within these regions that are devoid of blood-brain barrier. Although we have yet to determine whether ERα is colocalized within CD14- and TLR4-expressing cells, it is tempting to propose that interaction between E2 and ERα in the CVOs is required for a proper immune response to infection.

The ability of E2 to modulate the innate immune response is however not specific to the CVOs because centrally injected LPS is able to activate microglial cells in a sex steroid-dependent manner in female mice. Although in situ hybridization and immunohistochemistry may not be sensitive enough to detect ERα in microglia, E2 was found to modulate the effects of LPS on primary cultures of microglial cells (12). This effect is also the case for TLR4 transcript that is undetectable in microglial cells by in situ

FIGURE 5. Widespread viral replication and inflammation in the brain of OVX mice. Both intact and OVX groups were inoculated intranasally with 20 μl of MEM containing 10³ PFU of HSV-2. A. HSV-immunoreactive (ir) neurons in the hypothalamic paraventricular nucleus and the anterior/lateral hypothalamic area of OVX mice 10 days after the intranasal inoculation. B. Laser scanning confocal microscopic image of HSV-immunoreactive (ir) neurons (green, FITC) and the neuronal marker NeuN (red, Alexa Fluor 594). Laser scanning confocal microscopy studies were performed with a x20 and x100 Plan-Apo oil immersion objective, numerical apertures 0.8 and 1.35, ×2 numerical zoom with a BX-61 microscope (Olympus America). Emissions were recorded by photomultipliers preset, respectively, for FITC (green pseudo color) and Alexa Fluor 594 (red pseudo color) fluorescent dyes in Fluoview SV500 imaging software. Thirteen 0.5-μm confocal z-series were acquired for each area and corrected by two Kahlman low speed scans. Acquired z-series images were then flattened into one image and exported in 24 bit TIFF format. C. Expression of TLR2 (used as a general index of macrophage/microglia activation) mRNA during the course of HSV-2 replication in the brain of intact and OVX mice is shown. Coronal sections were taken from x-ray films (Biomax; Kodak). Note the strong immunoreactive signal for HSV-2 in the hypothalamic nuclei and areas of OVX animals (A, right column), but not in the rostral brain of intact mice inoculated with the virus (A, left column). These stressed neurons no longer contain nuclear NeuN staining (B, right column). A robust TLR2 transcriptional activation also took place in the mediobasal forebrain of HSV-treated mice devoid of estrogen (C, right column). Such a macrophage/microglial reactivity is a consequence of HSV-induced neuronal damage in the rostral brain of OVX mice (see Results).
hybridization (30), but these cells respond to LPS in a TLR4-dependent manner (26, 31, 32). It can therefore be concluded that LPS and E₂ can target their respective cognate receptors in microglial cells throughout the brain.

Interaction between LPS and TLR4 triggers signaling pathways that are similar to those activated by IL-1. Indeed, type II IL-1R has the same Toll/IL-1R homology domain capable of interacting with MyD88, an adapter protein that is recruited upon activation of type I IL-1R or TLRs. This leads to NF-κB signaling and transcriptional activation of numerous proinflammatory genes involved in the control of the innate immune process. A critical question at this point is how ERs interfere with this pathway and whether this effect is direct or indirect? ERα belongs to a superfamily of ligand-activated transcription factors comprising steroids, thyroid hormones, retinoids, and vitamin D characterized by a central DNA-binding domain capable of targeting the receptor to specific DNA sequences known as hormone response elements (33). ERs has a modular structure consisting of distinct functional domains allowing the binding on the consensus site AGGTCA referred to as the estrogen response element. The consensus estrogen response element is present in a number of genes expressed in the brain, which are known to be under the influence of estrogen. In this regard, Wissink et al. (34) have shown that estrogen can up-regulate expression of the serotonin-1A receptor via a new mechanism involving synergistic activation by NF-κB with ERα. These authors have proposed that NF-κB complexes cooperate with ERα to recruit cofactors into the complex and thereby synergistically activate the promoter through nonclassical estrogen response elements by a mechanism that does not involve direct receptor binding to DNA (34). It would be interesting to investigate whether such a mechanism takes place in microglial cells in vivo.

It is nevertheless important to mention that numerous studies have provided evidence that estrogens inhibit NF-κB and suppress proinflammatory signaling and cytokine gene expression. Binding of E₂ to its nuclear ERα was found to interfere with cytokine-induced activation of a NF-κB reporter in HepG2 cells (35). Transcriptional activity of the RelA subunit of NF-κB can be suppressed in a hormone-dependent manner (36, 37), whereas 17β-E₂ is able to decrease LPS-induced NF-κB binding activity and IL-1α, IL-6, and TNF-α production, but not IL-10, IL-12, and MIP production by splenic macrophages (38). Inducible NO synthase expression is attenuated by E₂, though the latter failed to alter NF-κB activation in primary rat microglia and N9 microglial cell lines (15). Ospina et al. (39) have reported that estrogen decreases the inflammatory response to IL-1 in the brain in OVX animals and that sex hormone replacement in OVX rats attenuated LPS fever (40), but it potentiated IL-1β-induced fever and cyclooxygenase-2 expression in the brain (41). E₂ was also found to significantly prevent LPS-induced microglial reactivity in an ERα-dependent manner (13, 14). The latter studies reached these conclusions based on the changes in inducible NO synthase, PGE₂, and metalloproteinase-9 levels, which have not been assayed in the present study. It is therefore difficult to explain the discrepancies between these studies and the present set of data, but the effects of sex steroids may depend on the target genes. In the present series of experiments using various approaches and models in vivo, expression of genes involved in the control of the innate immune response was clearly attenuated in microglial cells of OVX mice challenged with a Gram-negative cell wall component.

Among the genes assessed in the present study, IL-12 is of particular interest. IL-12 is a key cytokine involved in the transfer between the innate and adaptive immunity. Macrophage-derived IL-12 stimulates the differentiation of a subset of T lymphocytes (CD4⁺) into Th1 cells that produce IFN-γ. These activated Th1 cells are actually believed to play a critical role in multiple sclerosis, especially during the demyelinating episodes (see review Ref. 42). Of interest is that multiple sclerosis occurs more commonly in females than males indicating that sex steroids may play a role in this autoimmune disease. Our data fit very well with this mechanism and the permissive properties of E₂ on the innate immunity may explain the susceptibility of females to autoimmune diseases. In contrast, alteration of circulating E₂ levels during cerebral infection may have profound consequences for the CNS if T cells are not properly activated by APCs in the brain microglia. The fact that these cells no longer express IL-12 mRNA in OVX mice treated with LPS clearly support the concept that appropriate transfer from innate to acquired immunity is dependent on estrogens in the female brain.

Interestingly, neurovirulence and neuronal damage spread into the regions of OVX mice not previously found in male (21) and intact female mice (as found in our study). Numerous hypothalamic areas and nuclei exhibited a strong immunoreactive signal for HSV only in OVX mice. These groups of infected and degenerating neurons were also associated with microglial reactivity as indicated by the robust hybridization signal for the gene encoding TLR2 in the mediobasal forebrain (Fig. 5). It can therefore be concluded that inappropriate transfer from innate to adaptive immunity allows HSV-2 to replicate more aggressively within neurons of the CNS. In this regard, deficient IFN-γ production was reported to be directly related to HSV-2 neurovirulence (43). The low IL-12 production by microglia/macrophages may then be a key mechanism for the pathogens to hide from the immune surveillance resulting in increased viral replication in the brain of OVX mice.

Estrogen replacement therapy has been associated with neuroprotection and the current dogma is that these effects are attributable to the immunosuppressive properties of these hormones. In this study we propose that it is actually the opposite. A defective innate immune response takes place in the brain of OVX mice in an ERα-dependent manner. This may render the host more vulnerable to infection in the presence of low circulating E₂ levels and exacerbate neuronal damages due to an inappropriate innate immune reaction by microglia. Obviously, these neuroprotective effects of estrogens are likely to differ between models in which proinflammatory cytokines are generally associated with adverse outcomes, such as in ischemia reperfusion injuries and chronic demyelinating diseases.

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Disclosures
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References


