

Minireview

**Shaping microglial phenotypes through estrogen receptors: Relevance to sex-specific
neuroinflammatory responses to brain injury and disease**

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Abbreviations:

AD, Alzheimer's disease; ADIOL, 5-androstenediol; ALS, amyotrophic lateral sclerosis; AP1, activating protein 1; BBB, blood brain barrier; BDNF, brain derived neurotrophic Factor; CC5, Cchemokine ligand 5; CNS, central nervous system; Cox-2, cyclooxygenase 2; DBD, DNA binding domain; DG, dentate gyrus; DPN, diethylpropionitrile; E, embryonic; E₂, estradiol; EAE, experimental autoimmune encephalomyelitis; EGFR, epidermal Growth factor receptor; ER α , estrogen receptor alpha; ER β , estrogen receptor beta; ERE, estrogen response element; GCI, global cerebral ischemia; GPR30, G protein-coupled receptor 30; Iba1, ionized calcium-binding adapter molecule 1; IGF-1, insulin like growth factor 1; INF- γ , interferon gamma; IL-1 β , interleukin 1 beta; IL-6, interleukin-6; IL-10, interleukin 10; iNOS, inducible nitric oxide synthase; LBD, ligand binding domain; LPS, lipopolysaccharide; MAC, macrophage antigen; MAPK/ERK, mitogen-activated protein kinase; MCAo, middle cerebral artery

occlusion; MHC II, major histocompatibility complex II; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory Protein 2; MMP-9, matrix metalloprotease 9; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MS, multiple sclerosis; NF- κ B, Nuclear factor kappa B; OGD, oxygen-glucose deprivation; OGD/R, oxygen glucose deprivation and reperfusion; OVX, ovariectomy; PD, Parkinson's disease; PI3K, phosphoinositide-3-kinase; PKA, protein kinase A; PPT, propyl pyrazole triol; SCI, spinal cord injury; SERMS, selective estrogen receptor modulators; STAT3, transducer and activator of transcription; T, testosterone; TBI, traumatic brain injury; TNF- α , tumor necrosis factor alpha; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4.

Abstract

In mammals, 17 β -estradiol (E₂), the primary endogenous estrogen, maintains normal CNS function throughout life and influences brain responses to injury and disease. Estradiol-induced cellular changes are mediated through the activation of nuclear and extranuclear estrogen receptors (ERs), which include ER α , ER β , and the G-protein coupled receptor, GPER1. ERs are widely expressed throughout the brain, acting as transcriptional effectors or rapidly initiating membrane and cytoplasmic signaling cascades in practically all brain cells including microglia, the resident immune cells of the CNS. Activation of ERs by E₂ exerts potent anti-inflammatory effects through mechanisms involving the modification of microglial cell responses to acute or chronic brain injury. Recent studies suggest that microglial maturation is influenced by the internal gonadal hormone milieu and that their functions in the normal and diseased brain are sex-specific. However, the role that each ER subtype plays in microglial development or in determining microglial function as the primary cellular defense mechanism against pathogens and injury remains unclear. This is partly due to the fact that most studies investigating the mechanisms by which E₂-ER signaling modifies microglial cellular phenotypes have been restricted to one sex or age. This review examines the different *in vivo* and *in vitro* models used to study the direct and indirect regulation of microglial cell function by E₂ through ERs. Ischemic stroke will be used as an example of a neurological disease in which activation of ERs shapes microglial phenotypes in response to injury in a sex- and age-specific fashion.

Significance Statement

As the primary immune sensors of CNS damage, microglia are important potential therapeutic targets. Estrogen receptor signaling modulates microglial responses to brain injury and disease in a sex- and age-specific fashion. Hence, investigating the molecular mechanisms by which estrogen receptors regulate and shape microglial functions throughout life may result in novel and effective therapeutic opportunities that are tailored for each sex and age.

Introduction

Microglial cells are responsible for the innate immune defense of the central nervous system (CNS). Upon sensing foreign stimuli, they undergo phenotypic changes that allow them to act as macrophages phagocytizing neurotoxins, dying cells and debris, and to initiate the neuroinflammatory signaling that protects and repairs damage (Salter and Stevens, 2017). Beyond surveillance, microglia are also key partners of neurons in maintaining normal CNS homeostasis. This is exemplified by their role in the activity-dependent refinement of neural circuits via synaptic pruning in both the developing and adult CNS (Ji et al., 2013; Paolicelli et al., 2011). The exquisite sensitivity of microglia to changes in the internal and external environments allows them to make essential contributions to the normal expression of a growing list of CNS functions, including learning and memory (Morris et al., 2013), motor function (Kana et al., 2019), and social (Smith and Bilbo, 2019) and sex behaviors (Lenz et al., 2013). Recent findings showed that microglia adapt to the changing demands of the developing and adult CNS in a sex- and age-specific fashion (Hanamsagar and Bilbo, 2016). Hence, impairment of microglial function will have different impacts on brain responses to injury and on the risk and manifestation of psychiatric and neurological disorders in men vs. women. In this regard, microglial responses to traumatic brain injury (TBI), ischemic stroke, and nociceptive stimuli, as well as the phenotypic changes they undergo during the course of neurological disorders such as Alzheimer's disease (AD) and Parkinson's disease (PD), are age- and sex-specific (Hanamsagar and Bilbo, 2016; Kerr et al., 2019). The gonadal steroid hormone estrogen is a major signal responsible for shaping developmental and adult sex-specific brain function in a manner that involves modulation of microglial phenotype.

The brain is a target of peripheral and locally produced 17β -estradiol (E_2), the primary endogenous estrogen. E_2 plays a key role in shaping brain architecture during development and is the principal driver of sexual differentiation in the rodent brain (McCarthy et al., 2018). Testicular production of androgens during embryogenesis (embryonic day; E 16-18) and shortly after birth, demarks critical time windows when the conversion of testosterone (T) to E_2 by brain aromatase allows the latter to exert permanent organizational effects. In contrast, embryonic and neonatal ovaries are largely inactive, and a

lack of exposure to E₂ is thought to drive normal female brain development. Hence, male and female brains have very different internal hormonal states during neurogenesis, neuronal migration, and synaptogenesis, and importantly, during microglial cell maturation. Starting at E 8.5, yolk sac progenitor microglia cells travel through the bloodstream to the brain until the blood brain barrier (BBB) is formed. Microglia then follow a gradual and precise developmental program in which colonization and proliferation are accompanied by time-specific changes in their morphology (from amoeboid in the embryonic brain to ramified in the adult brain), and in the ability to regulate or support specific processes such as axon guidance, synaptic patterning, cell genesis, phagocytosis of dead cells, and myelination (Lenz and Nelson, 2018). Thus, beginning in utero, and then postnatally, E₂ has the potential to shape microglial development, which might contribute to permanent sex-specific manifestations of normal or impaired brain functions.

E₂ also modulates adult microglial phenotypes. The best documented example of this is attenuation of microglial pro-inflammatory responses to brain injury (Azcoitia et al., 2019; Johann and Beyer, 2013). Although microglia play a vital role in protecting neurons, their prolonged hyper-activation results in the production of reactive oxygen species, cytokines, and proteases that kill neighboring cells and damage the BBB (Nissen, 2017). These pro-inflammatory responses are stronger in the aging brain, where microglia are already primed into a more reactive, pro-inflammatory phenotype (Lourbopoulos et al., 2015; Mangold et al., 2017a; Mangold et al., 2017b; Zoller et al., 2018). An activated pro-inflammatory microglial phenotype has also been linked to the development of chronic neurodegenerative diseases such as AD, multiple sclerosis (MS), PD, and amyotrophic lateral sclerosis (ALS) (Hanamsagar and Bilbo, 2016; Lenz and Nelson, 2018). E₂ treatment prevents or decreases expression of the microglial reactive (M1-like) state and promotes the more reparative (M2-like) state in several models of brain injury and neurodegenerative diseases (**Tables 1 and 2**). Among the functional reactions of microglial cells to E₂ are the complete inhibition or dampening of responses such as nitric oxide (NO) production, release of pro-inflammatory cytokines, cell migration and phagocytosis, as well as a reduction in the

expression of pro-inflammatory genes. Central to the mechanisms by which E₂ exerts these microglial cellular changes are the proteins to which it binds, the estrogen receptors (ER).

E₂ neuroprotective and anti-inflammatory actions are mediated by the activation of three ER subtypes: the classical ERs, ER α and ER β , and the membrane ER, GPER1 (also known as GPR30). Transgenic mouse models with global or cell-specific ER knock outs, as well as pharmacological tools such as selective ER agonist and antagonists, have been utilized to investigate the role that each ER subtype has in E₂ modulation of microglial phenotypes. However, the contribution of specific ER subtypes and the cellular mechanisms they employ to mediate the anti-inflammatory and hence neuroprotective effects of E₂ are still unclear. Even less is known about the role that each ER subtype might play in microglial development and maturation, and hence in determining the sex-specific functions of these cells. The present review examines the different experimental models used to study E₂ effects on microglia and the specific ER subtypes involved. It also appraises the research conducted on microglial responses to ischemic stroke, a sexually dimorphic neurological disease in which E₂-ER neuroprotective and anti-inflammatory actions are determined by both biological sex and age.

Estrogen receptors at a glance

The majority of studies investigating the effects of E₂ on microglial immunological responses focus on activation of the classical ERs, ER α and ER β . Classical ERs are members of the nuclear hormone receptor superfamily, and although encoded by distinct genes (ESR1 and ESR2, respectively) they share a multi domain protein structure that confers similar mechanisms of hormone response (Hewitt and Korach, 2018). Their ligand binding domain (LBD) confers affinity and specificity to E₂ whereas their DNA binding domain (DBD) confers binding to estrogen-responsive elements (ERE) in the DNA. In the classical model of ER signaling, ERs form homodimers or heterodimers in the presence of E₂, translocate to the nucleus, and trigger transcription by binding to EREs. Alternatively, ERs can interact with other transcriptional proteins such as activating protein 1 (AP1), signal transducer and activator of transcription 3 (STAT3), or nuclear factor kappa B (NF- κ B) to regulate transcription at non-ERE sites

(Hamilton et al., 2017). ER α and ER β can also work in a *non-classical* membrane-initiated manner and in a *ligand-independent* fashion. Plasma membrane-initiated signaling is triggered by binding of E₂ to either classical ER and leads to diverse and rapid cytoplasmic effects, such as the activation of the mitogen-activated protein kinases (MAPK/ERK) and the phosphoinositide 3-kinase (PI3k)-Akt signaling pathways (Hamilton et al., 2017; Hewitt and Korach, 2018). In addition, growth factors such as insulin like growth factor 1 (IGF-1) may bring about ligand-independent ER activation by inducing the phosphorylation of ERs; phosphorylated ERs may then bind to DNA and stimulate transcription. (Hewitt et al., 2017).

Estradiol's rapid non-genomic actions in the CNS can also be mediated by the activation of *non-classical* membrane ERs. Of these, the G protein coupled receptor, GPER1, is the best characterized (Alexander et al., 2017). GPER1 signals via the G α s protein to activate the protein kinase A (PKA) and ERK signaling pathways. GPER1 also couple to the pertussis toxin-sensitive G $\alpha_{i/o}$ protein, which can activate of the PI3K or the MAPK/ERK signaling pathways via the transactivation of the epidermal growth factor receptor (EGFR). In addition, GPER1 activation elevates intracellular Ca²⁺ levels through phospholipase C and inositol (1,4,5)-triphosphate receptors.

Both the classical ERs and GPER1 are distributed throughout the CNS, with ER α and ER β showing a sexually dimorphic, brain-region-specific distribution (Kelly et al., 2013; Zuloaga et al., 2014). These receptors are highly expressed in neurons (Hazell et al., 2009; Mayer et al., 2010; Zuloaga et al., 2014), astrocytes (Morgan and Finch, 2015; Platania et al., 2003; Spence et al., 2011) and oligodendrocytes (Khalaj et al., 2013; Platania et al., 2003) and ER signaling in these brain cells is diverse. Upregulation of neurotrophic and neuroprotective molecules such as brain derived neurotrophic factor (BDNF) and IGF-1, activation of cell survival pathways such as PI3K-AKT signaling, and suppression of apoptotic signaling, are all associated with membrane and classical ER-mediated neuroprotective actions of E₂ in brain cells (Azcoitia et al., 2019). The ER subtype activated by E₂ as well as the molecular mechanisms involved depend on the disease model and the brain-region under investigation.

E₂ also maintains normal CNS homeostasis and protects the brain against injury and disease through its potent anti-inflammatory effects in microglial cells. E₂ treatment transforms microglial cell phenotype from a reactive M1-like to a less reactive M2-like state during responses to various insults, such as lipopolysaccharides (LPS) or hypoxia (Johann and Beyer, 2013); **Tables 1, 2**). E₂ is likely to modulate microglial cell function through multiple mechanisms. As with other brain cells, the specific ER signaling pathway that mediate E₂ effects on microglia will depend on the disease model under investigation, brain region, gonadal hormone status, sex, and age.

Although the anti-inflammatory and neuroprotective actions of E₂-ER signaling are undisputed, we do not have a clear understanding of how E₂-ER signaling varies with age. This is an important gap in the field. A reduction in ER expression or function might explain, at least in part, the increased vulnerability of the brain to damage and the decreased E₂ neuroprotective effectiveness with age. Indeed, a decrease in the ratio of ER α :ER β expression in hippocampal and cortical neurons with age correlated with the inability of E₂ to facilitate memory in aged rats (reviewed in (Foster, 2012). In contrast, the ER α :ER β ratio in rat astrocytes increased with cessation of estrous cyclicity and with age in females, and correlated with the loss of E₂-dependent neurotrophic activity (Morgan and Finch, 2015). Less is known about changes in ER expression in the aging mouse brain. Some studies report a decrease in ER β protein (Sharma and Thakur, 2006) or mRNA (Thakur and Sharma, 2007) in the absence of changes in ER α expression in the cortex of aged female mice (Thakur and Sharma, 2007). On the other hand, a recent study reported a reduction in ER α and ER β protein expression in the hippocampus of old (20-months) vs. young female (3-months) mice (Zhao et al., 2017). As discussed below, aging and decreased gonadal hormones are associated with impaired microglial neuroprotective functions. Because the decrease in sex hormones after menopause is correlated with an increase prevalence of neurological disorders in women (Brinton et al., 2015), it will be important to examine whether changes in E₂-ER signaling contributes to microglial dysfunction.

E₂ anti-inflammatory effects in the CNS

Removal of the main source of E₂ production by ovariectomy (OVX) has been extensively used to study acute and long-term effects of this hormone on brain function and to examine the contribution of gonadal hormones to the sex differences observed in microglial phenotypes. Chronic (several weeks) as opposed to acute (5-14 days) OVX is also used to mimic the absence of endogenous hormone production experienced by women after menopause. In rats and mice, chronic OVX promoted the appearance of highly reactive microglia (Cordeau et al., 2016; Lei et al., 2003; Vegeto et al., 2006), exacerbated the induction of their M1-like pro-inflammatory state by immune challenges such as LPS (Vegeto et al., 2006; Wu et al., 2016), and accelerated the phenotypic pro-M1 changes associated with aging in mice (Wu et al., 2016). Most microglial phenotypic changes in response to LPS or injury are prevented by the chronic administration of E₂ when the hormone treatment starts at the time OVX or shortly thereafter (**Table 1**). For example, in young OVX rats, chronic administration of E₂ decreases the LPS-induced increase in the number of cortical and hippocampal microglia cells expressing matrix metalloproteinases 9 (MMP-9), a known contributor to neuronal injury (Vegeto et al., 2003). Similarly, the LPS-induced expression of chemokines (e.g., MCP-1 and MIP2) and pro-inflammatory cytokines (TNF- α) was reduced by chronic E₂ treatment in OVX mice or rats (Vegeto et al., 2006).

The anti-inflammatory actions of E₂ are also observed in animal models of injury or of neurological conditions characterized by cognitive and/or motor dysfunction such as stroke, TBI, spinal cord injury (SCI), PD, or AD. (**Table 1**). For example, E₂ treatment diminishes the activation, proliferation and migration of microglia towards sites of injury. This was demonstrated in the oculomotor nucleus after peripheral axotomy in OVX mice (Gyenes et al., 2010) and in the brain or spinal cord after TBI in both sexes (Barreto et al., 2007; Barreto et al., 2014; Sribnick et al., 2005). In the APP23 mouse model of AD, E₂ decreased the number of plaque-bearing activated microglia (Vegeto et al., 2006), and in a model of cuprizone-induced demyelination, E₂ delayed microglia accumulation and reduced mRNA expression of TNF- α (Taylor et al., 2010). However, E₂ treatment does not always fully restore the changes in microglial phenotypes induced by damage. For example, in a study using the toxin 1-methyl-

4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to model PD, E₂ treatment prevented microglial cell proliferation without affecting morphological changes in microglia induced by MPTP (Tripanichkul et al., 2006). Varied effects of E₂ might be due to differences in the route, dosage, and/or timing of hormone administration. Most studies utilized E₂ treatments that result in physiological circulating levels (0.3 – 145 pg / ml, (Nilsson et al., 2015), while others utilized treatments that produce supraphysiological E₂ levels (Table 1 and 2). This is important, as the levels of E₂ can affect the regulation of ER subtype expression in a brain region- and cell type-dependent fashion. For example, chronic E₂ deprivation in rats decreased hippocampal ER α expression (Zhang et al., 2011) whereas treatments producing supraphysiological plasma E₂ levels in mice can decrease hippocampal ER α expression (Iivonen et al., 2006). We lack information about how changes in gonadal hormone levels in young and old animals affect ER subtype expression in many cell types, including microglia. Therefore, selection of the appropriate route, dosage and timing of E₂ administration to achieve physiological levels is critical to generate relevant and reproducible data.

Involvement of classical and membrane ERs in E₂ anti-inflammatory effects in the CNS

Few studies have focused on identifying which ER(s) is responsible for E₂ anti-inflammatory actions and its effects on microglia. However, activation of ER α signaling may play a key role. First, the anti-inflammatory actions of E₂ against LPS treatment were lost in mice carrying a global deletion of ER α but not ER β (Vegeto et al., 2003). Moreover, in transient transfection assays using a reporter for the MMP-9 gene promoter, E₂ activation of ER α , but not ER β , prevented MMP-9 promoter induction, suggesting that ER α transcriptional mechanisms play a role in E₂ anti-inflammatory effects. Subsequent studies using primary neonatal microglia cultures showed that E₂ inhibits LPS activation of NF-kB signaling through rapid non-genomic actions mediated through the PI3K signaling pathway (Ghisletti et al., 2005). These E₂ effects were absent in ER α -null microglia but not in WT or ER β -null microglial cells.

Selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene, which bind to both ER α and ER β , show that SERMS have neuroprotective actions that are often accompanied by a reduction in microglial reactivity (Baez-Jurado et al., 2019). For example, chronic E₂ or raloxifene treatment lowered the number of Mac-1 positive microglial cells in the dentate gyrus and CA1 hippocampal regions of aged (20-24 months) OVX mice (Lei et al., 2003). In addition, raloxifene or tamoxifen decreased expression of the major histocompatibility complex II (MHC II), a marker of antigen-presenting activity, in young and aged OVX rats in a model of TBI (Barreto et al., 2014). In other contexts, such as *in vitro* studies using microglial cells, these two SERMS were used to block the activation of ER α and ER β by E₂. Raloxifene and tamoxifen can exert anti-inflammatory and neuroprotective effects through the activation of GPER1 (Alexander et al., 2017). Therefore, caution should be taken in interpreting the protective and anti-inflammatory actions of SERMS, as they might involve the activation of both classical and non-classical ERs (**Table 1**).

Selective ligands for ER β or GPER1 have been investigated as alternatives to E₂ treatment for the improvement or prevention of microglial hyperactivation and other negative sequelae caused by brain injury or neurodegenerative diseases. In the experimental autoimmune encephalomyelitis (EAE) model of MS, treatment of affected animals with ER β -selective ligands such as the steroid hormone derivative 5-androstenediol (ADIOL; (Saijo et al., 2011), or the benzopyran LY3201 (Wu et al., 2013), reduced EAE clinical severity scores and mortality and decreased the macrophage/microglia activated state, as evidenced by downregulation of NF- κ B and inducible nitric oxide synthase (iNOS) expression in microglia. In a model of TBI, treatment of male rats with the GPER1 agonist G1, improved cognitive function and exerted neuroprotective effects (Pan et al., 2018). The beneficial effects of G1 in this TBI model were explained in part by a G1 induced decrease in neuronal apoptotic signaling, evidenced by a reduction in active caspase-3 positive hippocampal neurons, and by the promotion of a more M2-like anti-inflammatory microglial phenotype.

E₂ regulation of microglial phenotypes through ERs

To study the mechanisms underlying modulation of microglial phenotypes by E₂, immortalized microglial cell lines, primary cultures of neonatal rat and mouse microglia, and primary cultures of adult rodent microglia have been employed. These *in vitro* systems offer a controlled environment in which to determine the role that each ER subtype have in the E₂ modulation of microglial responses to challenges including LPS, hypoxia, amyloid β , and bacteria. (**Table 2**).

Immortalized microglial cell lines

BV-2 and N9 cells are widely used surrogates of primary microglia for *in vitro* studies. They were created by the retroviral transduction of oncogenes into mouse neonatal (BV-2) or mouse embryonic (N9) primary microglia (Stansley et al., 2012; Timmerman et al., 2018). Among the phenotypic characteristics they share with primary microglial cells are the expression of microglial markers such as macrophage antigen (MAC) 1 and MAC2, as well as LPS-induced production of nitric oxide and of the cytokines interleukin-6 (IL-6), TNF- α and interleukin-1 (IL-1), although the latter responses are relatively lower in the immortalized cells (Stansley et al., 2012). Pre- or co-treatment of these cells with E₂ suppressed the LPS-induced activated and pro-inflammatory M1 phenotype, and enhanced their anti-inflammatory M2 characteristics in a dose-dependent manner (Benedek et al., 2017; Thakkar et al., 2018). Moreover, treatment with the non-selective ER α/β antagonist ICI-162,673 blocks E₂ effects in both cell systems, suggesting the involvement of the classical ERs (Bruce-Keller et al., 2000). In fact, classical ERs are differentially expressed in BV-2 and N9 cells, with most studies showing that BV-2 cells exclusively express ER β , whereas N9 cells express both ER α and ER β mRNA and protein (**Table 3**). Studies investigating the effects of selective ER agonists have taken advantage of the selective expression of ER β in BV-2 cells. Treatment of the cells with the ER β -selective ligand diarylpropionitrile (DPN) reduced LPS-stimulated iNOS and cyclooxygenase -2 (Cox-2) expression, whereas the ER α -selective ligand propyl pyrazole triol (PPT) was without effect (Baker et al., 2004). More recently, opposing actions of ER β and GPER1 activation on the phagocytic activity of BV-2 cells were described; DPN enhanced but

the GPER1 agonist G1 attenuated the ability of microglia to phagocytose apoptotic PC12 cells (Loiola et al., 2019).

In N9 cells, E₂ treatment attenuated LPS-induced superoxide release and phagocytic activity in a dose-dependent manner, and these effects were blocked by the MAPK antagonist PD98059 or the ER α / β antagonist ICI 182 780 (Bruce-Keller et al., 2000). Other studies showed that pre-treatment of N9 cells with E₂ also increased the release of the anti-inflammatory cytokine interleukin-10 (IL-10) and decreased release of the pro-inflammatory molecules TNF- α and interferon gamma (IFN- γ) (Dimayuga et al., 2005).

Even though BV-2 and N9 cells are useful models to elucidate molecular mechanisms mediating E₂ effects, these immortalized cells are not identical to primary microglia. For example, they show a more adherent and proliferative phenotype as well as a different transcriptome profile (He et al., 2018; Stansley et al., 2012). Moreover, upon examining the expression of the Y chromosome-specific gene *SRY*, Crain and Watters (Crain and Watters, 2010) found that BV-2 cells are female and N9 cells are male. Previous studies had not considered the sex of these cells. Hence, it should not be assumed that the results obtained using these cells can be generalized to other injury models in which microglial participate.

Primary microglial cultures

The immunoregulatory effects of E₂ in models utilizing primary microglial cells harvested from neonatal or adult rodents are diverse (**Table 2**). As with their immortalized counterparts, E₂ treatment of primary microglia prevents the morphological changes and production of pro-inflammatory signals induced by diverse toxic challenges such as LPS and hypoxia. However, there are contradictory reports about the roles ER subtypes play in the direct actions of E₂ on microglia *in vitro*. Some studies reported low but detectable mRNA expression of ER α but not ER β mRNA in primary cultures of neonatal mouse microglia (Crain et al., 2013; Sierra et al., 2008); **Table 3**). mRNA and protein expression of ER β and GPER1 but not ER α was reported in primary microglial cell cultures from neonatal rats (Habib et al., 2014); yet other investigators detected expression of both classical ERs in this system (Liu et al., 2005; Vegeto et al., 2001). Similar discrepancies are found when reviewing studies using primary cultures of

adult rat or mouse microglia (**Table 3**). However, until recently it was not considered that microglia might show sex-specific characteristics *in vitro* or that neonatal microglia might behave differently.

Also influencing microglia specific ER expression are the isolation method used and whether the cells are harvested from whole brains or from a specific brain region. For example, it was reported that the gene expression in cultured microglial cells is significantly different from that of microglia freshly isolated from animals of the same age (Crain et al., 2013). In this regard, ER α mRNA levels in cultured primary microglia cells were significantly lower than in freshly isolated male and female microglia of any age. Follow-up studies showed that microglial gene expression of neuroprotective and pro-inflammatory genes varied by sex, age and the CNS region from which the microglia were obtained (Crain and Watters, 2015). In male mice of all ages examined, expression of ER α mRNA was significantly higher in microglia isolated from the cortex than the brainstem/spinal cord; in females, the difference in ER α mRNA was significant only at 12 months of age. Therefore, differences in ER subtype expression that occur as a result age, sex, tissue source, or method of microglia cell isolation may contribute to the diverse and often conflicting findings about the modulation of microglial function by E₂-ER signaling. In support of this idea, sex-specific effects on IL-1 β mRNA expression after LPS-stimulation and/or E₂ treatment were reported in both neonatal and adult rat primary microglia cultures (Loram et al., 2012). When using neonatal microglia, the males had a greater IL-1 β response to LPS compared to females. Co-administration with E₂ suppressed IL-1 β mRNA in males, whereas in females, IL-1 β mRNA was potentiated by E₂. The effects of LPS and of E₂ on IL-1 β mRNA was also sex-specific in microglia isolated from adult males or OVX female rats. In this case, the LPS-induction of IL-1 β mRNA was greater in males than in females and co-administration of E₂ suppressed IL-1 β mRNA in female but not male microglia. In contrast, E₂ treatment *in vitro* potentiated IL-1 β mRNA response to LPS in microglia isolated from OVX females that were supplemented with E₂ *in vivo*. Therefore, maturity of microglia at the time of isolation influences pro-inflammatory responses as well as E₂ effects on microglia phenotypes.

Because the vast majority of studies have utilized microglial preparations of mixed sex and/or neonatal microglial cultures, our understanding of ER subtype-specific actions on microglia remains incomplete.

Discrepancies in microglial specific expression of ER subtypes are also encountered in data from various *in vivo* experiments (**Table 3**). E₂ treatment *in vivo* alters microglial phenotypes in brain areas such as the cortex and hippocampus where expression of ER α and/or ER β is low. In a study using electron microscopic immunohistochemistry, sparse cytoplasmic ER α protein expression was reported in adult mouse hippocampal microglia (Sierra et al., 2008). In the rat cerebellum, punctiform ER α but not ER β immunostaining was observed in the soma and cell processes of microglia, and treatment with LPS, E₂, or tamoxifen increased ER α punctiform immunostaining in the microglial perikaryon (Tapia-Gonzalez et al., 2008). However, other studies detected ER β but not ER α protein expression in microglia or found that microglia express both classical ERs, albeit at very low levels (**Table 3**). As with studies investigating microglial cell-specific ER α/β mRNA, regional heterogeneity in CNS microglial ER expression, sex differences, and low ER α/β co-expression might explain these discrepancies. Interestingly, GPER1 recently emerged as an important mediator of E₂-induced anti-inflammatory effects on microglia. GPER1 is expressed in primary microglial cultures from neonatal rats and mice (Zhang et al., 2018; Zhao et al., 2016) and in adult murine primary microglial cultures (Loiola et al., 2019) and GPER1 protein has been detected in cortical microglia of female rats and mice (Zhang et al., 2018; Zhao et al., 2016). However, whether microglial expression of GPER1 is sexually dimorphic is not known.

These findings suggest that regulation of microglia cell functions by E₂-ER signaling cannot be excluded. Nonetheless, ER signaling in neurons and astrocytes likely participates in E₂ regulation of microglia reactivity to injury and disease, as the reciprocal communication among the three cells types is well established (Jha et al., 2019; Szepesi et al., 2018). Neurons release immune-related soluble factors that bind to their putative receptors on microglia and promote specific microglial changes (Qin et al., 2019). Neurotrophins, neuropeptides, neurotransmitters, anti-inflammatory cytokines and chemokines are among the many factors whose expression and release by neurons can be modified by estrogens through

direct activation of ERs. Neurotrophins such as neurotrophin-3 (NT-3), BDNF, and nerve growth factor (NGF), released by activated neurons, regulate microglial MHC-II expression and induction of pro-inflammatory molecules (Neumann, 2001). Receptors for these neurotrophic factors co-localized with ERs, and E₂ not only regulated the sensitivity of neurons to neurotrophins but also neurotrophin expression and release (Chan and Ye, 2017). Similarly, activation of ERs such as ER α in astrocytes could modulate the release of molecules that in turn regulate microglial phenotypes and functions such as motility and phagocytosis. Therefore, because the activity of one cell type influences the others, E₂-ER signaling in neurons or astrocytes might influence microglial phenotypes.

Role of ER signaling in sex-specific microglial responses to ischemic brain injury

Ischemic stroke, caused by a loss of blood flow to the brain, accounts for more than 87% of all strokes (Benjamin et al., 2017). Recent analysis of temporal trends suggests that stroke incidence maybe declining to a greater extent in men than in women, and that the overall decline in stroke incidence observed over time is being driven by a decrease in men (Madsen et al., 2017). The factors underlying this epidemiological phenomenon are unclear. However, sex and age are important non-modifiable variables affecting risk, pathophysiology, treatment responses, and outcomes in stroke (Bushnell et al., 2018; Roy-O'Reilly and McCullough, 2018). Fluctuations in the levels of sex hormones such as E₂ that occur throughout a women's lifespan, contribute to sex differences in stroke. For instance, menopause increases the risk of stroke in women, and early menopause is associated with a two-fold increase in risk for ischemic stroke (Rocca et al., 2012). Even though our understanding of E₂ neuroprotection in stroke has grown considerably, is not possible to incorporate the use of this or other sex hormones in the clinic to prevent or treat stroke. Concerns about increasing the risk for certain gynecologic cancers (Collaborative Group on Hormonal Factors in Breast, 2019) and the potential of E₂ to exacerbate stroke incidence and severity if given to elderly women (Wassertheil-Smoller et al., 2003), partly explain the lack of E₂ utilization as a therapeutic strategy for stroke. Moreover, in pre-clinical and clinical studies, promising therapeutic targets for stroke are often effective in males but not females (Amiri-Nikpour et al., 2015; Hagberg et al., 2004).

Activation of microglial cells is a key component of the post-ischemic inflammatory response that contributes to the extent of brain injury (Qin et al., 2019). Once ischemia occurs, microglia are activated to produce both detrimental and neuroprotective mediators, and the balance of the two counteracting mechanisms determines the fate of injured neurons (Benakis et al., 2014). Therefore, treatments that minimize the detrimental effects and/or maximize the protective actions of microglia have enormous clinical potential. In this regard, the antibiotic drug minocycline, which is commonly used to inhibit microglial activation (Lenz et al., 2013), has been touted as a promising neuroprotective agent in acute stroke patients. However, in a clinical study of minocycline neuroprotection in ischemic stroke, male but not female stroke patients who received oral minocycline daily for five days had significantly better neurological outcomes (Amiri-Nikpour et al., 2015). The reasons for the lack of effectiveness of minocycline and other drugs in women are not clear. However, cumulative evidence suggests that microglial responses to stroke injury are sex-specific (McCullough et al., 2016), developmentally determined (Villa et al., 2018), modified by age (Yan et al., 2014), and influenced by reproductive experiences such as pregnancy and parity that are unique to women (Ritzel et al., 2017). Because the expression and function of ERs is also developmentally regulated (Perez-Alvarez et al., 2012; Platania et al., 2003; Prewitt and Wilson, 2007; Wilson et al., 2011) and influenced by age (Ianov et al., 2017; Morgan and Finch, 2015; Zuloaga et al., 2014) and sex (Kelly et al., 2013; Waters et al., 2015; Wilson et al., 2011; Zuloaga et al., 2014) it is reasonable to propose that classical and membrane ERs participate in the manifestation of microglial responses to ischemic injury in a sex-and age-specific fashion.

Developmental programming of sex-specific microglial responses to ischemic injury

That the sex of microglia influences the progression of ischemic stroke was recently demonstrated by Villa et al. (2018) in a study in which the progression of brain damage induced by permanent middle cerebral artery occlusion (pMCAo) was monitored in male brains transplanted with purified male (M/M) or female (F/M) microglia (Villa et al., 2018). The progression of ischemic stroke was found to be more damaging in the M/M group than in the F/M group. The transplanted microglia accumulated in the vicinity of the infarct site, and the expression of the microglial anti-inflammatory

marker Ym1 was higher in male brains transplanted with female microglia. Significant differences in the transcriptomes of microglia isolated from the brains of healthy adult male and female mice suggest a neuroprotective phenotype of female microglia. Most of the genes that were highly expressed in male microglia are associated with inflammatory processes, such as regulation of cell migration and cytokine production. Moreover, whole-genome molecular signature analysis of transcription factors, identified NF- κ B as the transcription factor most involved in the regulation of the differentially expressed genes preferentially expressed in male microglia. In contrast, microglial genes expressed in female-derived cells were linked to transcription factors involved in the inhibition of inflammatory responses and promotion of repair mechanisms, including ER α -regulated pathways. The sex differences in adult microglia gene expression were proposed to originate from perinatal exposure to sex hormones, as treatment of female pups with E₂ eliminated the sex differences in genes highly expressed in male microglia. Importantly, quantitative analysis of brain ERE-Luc expression (a surrogate of ER α activity) showed that perinatal activation of ER α is restricted to males. Hence, the sex of microglia might be determined early in development through a mechanism that involves ER α signaling.

Similarly, Thion and colleagues (Thion et al., 2018) recently reported that microglia start expressing gene products that sense ligands and pathogens, also known as “sensitive” genes, in utero, acquiring a sexually dimorphic transcriptomic profile during the postnatal period. However, in contrast to the results of Villa, et al., female microglia were found to have a higher expression of genes associated with inflammatory and apoptotic responses than male microglia. In addition, using germ-free mice, this group found that the maternal microbiota impacted the microglial transcriptome in a temporal and sex-specific fashion. For example, absence of the maternal microbiome most severely affected embryonic microglia from males, whereas in females the most marked perturbations were observed in microglia from adults. Recent studies suggest that perturbation of the gut microbiota or dysbiosis influences the neuroinflammatory responses to ischemic injury (Singh et al., 2016). Whether perturbations in microbiota also influence microglial responses to ischemia in a sex-specific manner awaits examination.

ER signaling and microglial responses to ischemia in the aging brain

A large number of pre-clinical studies indicate that E₂ is neuroprotective in models of ischemic injury such as permanent or transient middle cerebral artery occlusion (MCAo; (Dubal and Wise, 2001) and global cerebral ischemia (GCI; (De Butte-Smith et al., 2009; Traub et al., 2009). For instance, female rodents were less susceptible to ischemic stress than males (Dang et al., 2011; Liu et al., 2009; Manwani et al., 2013). OVX prior to stroke induction abolishes this sex difference and treatment of males or OVX females with physiological doses of E₂ restores neuroprotection (Gibson et al., 2006; Petrone et al., 2014). The effectiveness of neuroprotection and the mechanisms utilized by E₂ to protect against ischemic brain injury varies with age and in the case of females whether E₂ is neuroprotective or neurotoxic is also determined by how long the brain has been deprived of E₂ (Dubal and Wise, 2001; Lewis et al., 2012; Liu et al., 2012; Selvamani and Sohrabji, 2010; Zhang et al., 2009). This age dependence of E₂ actions is significant in view of the increased vulnerability to stroke experienced by the elderly, especially elderly women (Scott et al., 2012).

The decreased of E₂ in protecting the brain with age is correlated with changes in ER expression. Expression of ER α splice variants that show dominant-negative regulation of ER α increased in the brain of elderly women (Ishunina and Swaab, 2008, 2009). Similarly, aging and /or extended E₂ deprivation caused a reduction in ER α , ER β and GPER1 in the hippocampus of female rats (Ianov et al., 2017; Wang et al., 2019; Zhang et al., 2011). Intriguingly, G1 was reported to reverse the reduction of ER α , ER β , and GPER1 expression within the hippocampus of aged female rats without affecting plasma E₂ levels (Wang et al., 2019). Studies of changes in cortical ER β and ER α expression with age in mice are scant, with one study reporting a decrease in ER α mRNA but not protein expression in the cortex of aged mice (Dietrich et al., 2015). Another study reported a significant decrease in ER β but not ER α protein in the mouse cortex with aging (Sharma and Thakur, 2006). Although it is clear that important changes in ER subtype expression occur with age, studies analyzing ER expression with cellular and neuroanatomical resolution

are missing. Experiments using validated antibodies for each ER subtypes and of microglial cell-specific markers in combination with novel reporter mouse models are needed to fill this gap.

Supporting the idea that loss of ER expression or function may underlie alterations in immune responses and increased vulnerability to stroke, Cordeau et al. (Cordeau et al., 2016) showed that chronic OVX of ER α null mice significantly altered the microglial activation profile and the innate immune responses after cerebral ischemia. This was shown by a significant attenuation of signals produced by the activation of the scavenger receptor Toll-like receptor 2 (TLR2) signaling in OVX ER α knockout animals after stroke. In contrast, chronic OVX of WT or OVX ER β null mice produced the expected increase in TLR2 signaling before and after stroke. Further analysis associated the attenuation of the TLR2 signaling in OVX ER α null females with markedly altered profiles of activated microglial cells, selective overexpression of IL-6, and over-activation of the JAK/STAT3 pathway as well as significantly larger ischemic lesions.

Little is known about the role of microglia cell-specific ER α signaling after ischemia. In one study, the role of neuronal vs. microglial ER α in E₂ neuroprotective effects was investigated using mice with a neuron- or myeloid cell-specific ER α deletion subjected to permanent MCAo (Elzer et al., 2010). The infarct volume of E₂-treated females lacking ER α in microglia/macrophages was similar to that of E₂-treated WT mice; in contrast, both male and female neuron-specific ER α -null mice had larger infarcts than control mice after E₂ treatment. It was concluded that neuronal but not microglial ER α signaling in mice of both sexes mediates neuroprotective effects of E₂ in this model of stroke. In this model, E₂ neuroprotective actions were tested using young males and young OVX females that received E₂ replacement at the time of surgery. It is unknown whether loss of microglial ER α or other ERs leads to cellular dysfunction and a higher vulnerability to ischemic injury in aged animals. This question is relevant and significant because sex- and/or aging-dependent changes in microglial activation profiles may be responsible for the observed increase in vulnerability to brain ischemia in elderly women.

ER β and GPER1 ligands: alternatives to decrease post-ischemic reactive microglial responses

According to the “critical period hypothesis”, E₂ therapy is beneficial for stroke and other brain diseases when taken by young women or older women during the peri-menopausal or early post-menopausal periods, but deleterious when taken by women who have been in menopause for many years (Scott et al., 2012). To circumvent concerns about the increased risk of breast and uterine cancers caused by E₂ treatment (Collaborative Group on Hormonal Factors in Breast, 2019), ER β agonists with minor effects in reproductive tissues, or of GPER1 ligands which exert non-genomic effects, have been investigated in the context of ischemic stroke and other CNS disorders.

ER β -selective ligands such as DPN were neuroprotective in various stroke models, such as GCI (Ma 2016) and transient focal ischemia (Shin 2013). DPN neuroprotective effects were associated with a decrease in astrocyte reactivity in OVX mice subjected to GCI, whereas in the focal stroke model DPN effects included a reduction in BBB breakdown. A recent study found that DPN treatment of eight-month-old OVX mice alleviated ischemic brain injury after MCAo and reperfusion and inhibited the activation of microglia and astrocytes in the ischemic penumbra (Guo et al., 2019). DPN treatment suppressed the protein levels of NF- κ B and of the pro-inflammatory cytokines, TNF- α , IL-1 β , and IL-6. Moreover, pretreatment of N9 cells with DPN increased cell viability and decreased NF- κ B and pro-inflammatory cytokine expression after oxygen-glucose deprivation and reperfusion (OGD/R), an *in vitro* model of global ischemia. These studies suggest that ER β -mediated neuroprotection against ischemic injury is mediated via multiple cell mechanisms, including a reduction in microglial reactivity and inflammation.

GPER1 may also participate in the neuroprotective actions of E₂ in focal and global ischemia. For instance, G1 protected against hippocampal neuronal loss induced by ischemia in middle-aged female rats (Lebesgue et al., 2010). In a model of focal ischemia-induced by MCAo, treatment of young OVX mice with G1 or E₂ reduced infarct volume and improved neurological deficits (Zhang et al., 2018). The effects of G1 included a reduction in penumbral production of TNF- α , IL-1 β , and IL-6, reduced co-expression of Toll-like receptor 4 (TLR4) protein in microglia, and inhibition of NF- κ B activity. The effects of E₂ were not altered by co-treatment with the ER α / β antagonist, ICI 182 780, suggesting that GPER1 plays a key neuroprotective role in this model. The anti-inflammatory effects induced by GPER1 activation were also

observed in primary microglial cultures subjected to OGD/R. In this *in vitro* model, a reduced release of TNF- α , IL-1 β , and IL-6, decreased in TLR4 mRNA levels and inhibition of NF- κ B activity by G1 were reported.

In summary, results from various experimental models of ischemic stroke suggest that E₂-ER signaling modifies the microglial pro-reactive state to suppress neuroinflammation and protect the brain. Anti-inflammatory E₂ effects are often accompanied by morphological changes in and decreased of microglia. Both *in vivo* and *in vitro* models show that activation of all major ERs ameliorates microglial cell responses to ischemic injury. Inhibition of the NF- κ B pathway and the concomitant reduction in microglial pro-inflammatory cytokine production play a major role in ER-mediated effects. E₂-ER signaling also modulates the expression of TLR2 and TLR4 receptors, which could impact microglial phagocytosis.

Concluding Remarks

Increasing evidence suggests that the role of each ER subtype in shaping microglial phenotypes is influenced by the internal gonadal hormone milieu during development, which in turns determines sex- and age-specific responses to injury in the adult brain. However, to further advance our understanding of E₂-ER signaling in microglial cells, we need to: **1)** Utilize novel genetic tools that can enable a comprehensive characterization of ER expression and function in microglial cells *in vivo*; **2)** Expand our investigations of microglial cellular responses to E₂ beyond that of their inflammatory phenotypes; and **3)** Employ *in vivo* and *in vitro* models that reflect the physiological changes in peripheral and local E₂ levels that occur with age.

One obstacle impeding the investigation of specific functions of microglia in CNS diseases has been the lack of markers that can distinguish resident microglia from infiltrating myeloid cells. The identification of the transmembrane protein 119 (TMEM119) as a specific marker of human and mouse microglia (Bennett et al., 2016), has enabled the generation of a specific antibody (Bennett et al., 2016) and a reporter mouse line (Ruan et al., 2020) that can be utilized to assess ER subtype expression in microglia with greater specificity. Discrimination between microglia and infiltrating macrophages could

be achieved through double immunofluorescence, utilizing Iba1 (ionized calcium-binding adapter molecule 1; a microglia/macrophage marker) in addition to TMEM119 antibody (Gonzalez Ibanez et al., 2019). Moreover, the research community has yet to utilize validated Cre mouse lines that specifically target microglia to study ER subtype function. The Cre models used up to now, not only lack microglial cell-specificity but also may have had a low recombination efficiency (Wieghofer et al., 2015). Complementary *in vitro* studies using primary cultures and co-culture systems may reveal novel mechanisms underlying E₂-ER effects on the microglial M1-like and M2-like state. As one example, changes in mitochondrial function in microglia in response to *in vitro* challenges such as OGD/R could give insight into the mechanisms that control E₂-ER-mediated changes in microglia anti-inflammatory or pro-inflammatory phenotypes. This is important, as strategies that drive microglia towards a neuroprotective phenotype are being sought to treat injury and neurodegenerative diseases. The value of such findings will depend on how well the models reflect physiological changes in E₂ levels that occur throughout life in both sexes.

That microglia are key targets of E₂ neuroprotective actions is undeniable. However, we are far from understanding how ER signaling in microglia varies with age and hormonal cycles in females, and these variables are known to impact microglial responses to injury and disease. Examination of the literature suggests that ER α signaling has a key role in determining the sex-specific microglial transcriptome. In addition, judging by studies using global knockout mice, ER α is also important in mediating E₂ anti-inflammatory effects and hence neuroprotection against ischemic injury and other neurological diseases. On the other hand, activation of ER β and GPER1 recruits alternative pathways by which E₂ protects the brain by decreasing microglial hyper-reactivity, which if unchecked is neurotoxic. Recent studies suggest that membrane and classical ERs interact with one another to enhance E₂ actions. For example, GPER1 required ER α / β to increase BDNF levels and to protect dopaminergic neurons in a mouse model of PD (Bourque et al., 2015). Adding to the complexity of ER-mediated pathways is the fact that changes in ER expression or function with age vary by cell type and hormonal status. It is

unknown whether microglia themselves experience changes in the expression or function of the different ERs with age, or if altered ER expression in other cell types impacts microglial function. Novel genetic and pharmacological tools to monitor and manipulate the expression of ER subtypes in microglial cells are needed to answer these questions.

According to recent population projections, the number of people over 60 years of age will more than double by 2050, and the number of people over 80 years old expected is expected to triple over this period (Nations, 2019). Because of the close relationship between age and the incidence of neurological diseases such as stroke, the increase in elderly people will be accompanied by a rise in the number of patients affected by these brain diseases is also expected to rise (Bejot et al., 2019). To better prepare for this challenge, it will be important to consider additional clinically relevant factors in the design of preclinical models such as those for ischemic stroke. For example, age-comorbid human conditions such as impaired metabolic control should be incorporated in experimental models of stroke and other brain diseases. In this respect, obesity and metabolic syndrome are suspected to play a role in a recent surge in mid-life stroke incidence among women (Peters et al., 2014). Therefore, understanding the mechanisms that regulate ER signaling with age in the presence of these and other co-morbid conditions is critical in order to identify effective therapeutics targeting microglia.

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Table 1. Examples of E₂ anti-inflammatory effects in *in vivo* models of CNS injury and disease. Studies reported the reactive state of microglia utilizing morphological assessments, expression of M1/M2 markers, or expression of cytokines and chemokines in the affected brain regions. Results might also reflect changes in the number of infiltrating macrophages to lesioned sites. Whether a physiological or pharmacological dose of E₂ was administered is also indicated; dose description is based on measurements of circulating E₂ levels reported by the studies or cited literature.

In vivo CNS disease model	Major effects	Dose	References
Intracerebral injections of LPS to young (6 weeks) OVX rats and to intact young adult ER α KO and ER β KO female mice.	E ₂ treatment decreases the number of microglia positive for MMP-9 in the cortex and hippocampus	SC: 0.5, 5.0 μ g/kg, physiological & 50 μ g/kg, pharmacological in ER α KO	(Vegeto et al., 2003)
Intracerebral LPS injection to young (6 weeks) OVX rats or C57/B6 mice. APP23 mice: Alzheimer's disease model; 5 mo old	Chronic or acute administration of E ₂ reduces the number of ED-1 (CD68) positive cells in the cortex and hippocampus of OVX rats; pre-treatment with E ₂ limits the mRNA expression of MIP-2, MCP-1, and TNF- α in the cortex and hippocampus of mice. Chronic E ₂ treatment decreases microglia activation (plaques bearing activated microglia using Mac-1 antibody).	SC: 50 μ g/kg Pellets: 0.025 mg/d for rats; 0.010 mg/d for mice.	(Vegeto et al., 2006)

females were OVX and then analyzed at middle aged (10-14 mo) mice were either ovx or sham operated.		Pellets: 0.01 mg/d achieving physiological (20-35 pg/ml) E ₂ levels	
Laminectomy in males: model of acute SC injury.	High doses of E ₂ post-injury reduces edema and decreases inflammation, shown as a reduction of CD11b/c (OX42) and ED-2+ cells.	IV: 4 mg/kg, pharmacological.	(Sribnick et al., 2005)
OVX of aged mice (20-24 mos.)	Chronic E ₂ or raloxifene treatment lowers the number of microglia cells (Mac-1 positive) in the DG and CA1 regions of the hippocampus.	Pellet: 1.7 mg, achieving physiological E ₂ levels (5.1 pg/mL)	(Lei et al., 2003)
MPTP injection in male mice: Parkinson's disease model.	Pre-treatment with E ₂ reduces of GSI-B4 positive microglia total number in the SNpc and striatum; lack of effect in GSI-B4 positive cell morphology.	SC: 2 µg/day, physiological.	(Tripanichkul et al., 2006)
TBI in male rats	E ₂ decreases hippocampal volume fraction of MHC-II immunoreactive microglia.	SC: 1 mg/kg, pharmacological.	(Barreto et al., 2007)

Intraperitoneal injections of LPS using males and OVX female rats.	Administration of E ₂ to male and OVX female rats dose dependently decreases the number of MHC-II immunoreactive cells in the cerebellum; cerebellar microglia from E ₂ treated animals also show longer and thinner cellular processes and smaller cell somas.	IP: 50, 250, 500 & 700 µg/kg, pharmacological	(Tapia-Gonzalez et al., 2008)
LPS treatment of OVX	Replacement with estrogen benzoate <i>in vivo</i> does not prevent LPS-induced changes in the mRNA expression of IL-6, TNF- α or TGF β 1 in microglia sorted from OVX females.	SC: 5 µg of estradiol benzoate, achieving high physiological E levels (126 pg/mL)	(Sierra et al., 2008)
Cuprizone-induced demyelination in male mice: MS model	E ₂ treatment delays microglia accumulation and reduces the mRNA expression of TNF- α in the corpus callosum.	Pellets: Achieving pregnancy levels (3000 pg/ml)	(Taylor et al., 2010)
TBI in young (2 mos.) and aged (18 mos.) OVX female rats	Raloxifene and Tamoxifen reduce microglia activation (volume fraction of MHC-II ⁺ microglia)	SC: 100 µg/kg, pharmacological	(Barreto et al., 2014)

Peripheral axotomy in OVX mice	Daily E ₂ treatments reduces microglia reactivity assessed by morphometric analysis of CD11b immunoreactive structures within the oculomotor nucleus.	SC: 50 µg/kg, supraphysiological	(Gyenes et al., 2010)
EAE MS model female mice	Treatment with the selective ERβ ligand LY3201 reduces iNOS expression in microglia	Pellet form: 0.04 mg/day	(Wu et al., 2013)
Sciatic nerve ligature: a model of neuropathic pain in male and females	Post-injury injection of E ₂ decreases neuropathy-induced microglia/macrophage hyperactivation.	SC: 50 µg/kg /day achieving high physiological E ₂ levels (50 pg/mL)	(Vacca et al., 2016)
OVX-induced microglia activation in aged mice (9 mos.) and LPS-induced microglia activation in young (3 mos.) males.	Chronic (3 mos.) E ₂ treatment antagonizes the OVX-induced changes in the SN microglia number and size, and on TLR4, p38, and TNF-α protein levels. Male mice pretreated with E ₂ show attenuation in the LPS-induced increases in Iba1 positive cells in the SN, striatum, hippocampus, and motor cortex.	Alzet osmotic mini-pump: 36 µg of E ₂ IP: 10 and 100 µg/kg	(Wu et al., 2016)

SOD1(G93A) males: ALS model	Treatment with E ₂ reduces microglial cell numbers in the SC. Expression of NLRP3 inflammasome proteins and levels of activated caspase 1 and mature IL- β were reduced in SOD1 males supplemented with E ₂ .	Pellet: 0.011 mg, 0.524 μ g/day, physiological.	(Heitzer et al., 2017)
GCI in young adult OVX rats	E ₂ treatment modifies microglia morphological changes in the hippocampus after GCI, from their “activated,” amoeboid morphology to a “resting,” ramified morphology and enhances the expression of anti-inflammatory microglia markers.	Alzet mini pumps: 0.0167 mg, achieving physiological levels (10-15 pg/mL).	(Thakkar et al., 2018)
SC injury in males	E ₂ treatment after injury attenuates microglia reactivity; this was accompanied by attenuation in the expression of certain inflammasome components.	SC: 25 μ g/kg, results in supraphysiological E ₂ levels (> 1,000 pg / mL).	(Zendedel et al., 2018)

MCAO in OVX mice	Treatment with E ₂ , G1, or E ₂ + ICI 182 780, immediately upon reperfusion reduces infarction volume, improves neurological deficits, and alleviates neuronal injuries. Any of these treatments also reduces the release of TNF- α , IL-1 β , and IL-6 from ischemic penumbra, Iba1 and TLR4 protein expression and inhibits NF- κ B activity.	IP: 235 nM	(Zhang et al., 2018)
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DG, dentate gyrus; E₂, 17 β -estradiol; EAE, experimental autoimmune encephalomyelitis; ER α , estrogen receptor alpha; ER β , estrogen receptor beta; GCI, global cerebral ischemia; GSI-B4, Griffonia simplicifolia B4 isolectin; Iba1, ionized calcium-binding adapter molecule 1; IL-6, Interleukin-6; iNOS, inducible nitric oxide; IP, intraperitoneal; IV, intravenous; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; MCP-1, monocyte chemoattractant protein-1; MHC-II, major histocompatibility complex II; MIP-2, macrophage inflammatory protein 2; MMP-9, matrix metalloprotease 9; MPTP, 1-methyl-4-phenyl 1,2,3,6-tetrahydropyridine; MS, multiple sclerosis; OVX, ovariectomy; SC, spinal cord and subcutaneous; SN, substantia nigra; SNpc, substantia nigra pars compacta; TBI, traumatic brain injury; TGF β 1, Transforming growth factor beta 1; TLR4, Toll like receptor 4; TNF- α , Transforming growth factor alpha.

Table 2. Examples of direct anti-inflammatory effects of E₂ in microglia *in vitro* models.

<i>In vitro</i> system	Challenge	ER ligand	Dose	Principal findings	References
BV-2 cells	LPS	E ₂	1 nM	Pre-treatment decreases LPS-induced NO production and the expression of iNOS and COX-2; increases LPS-stimulated TNF- α mRNA levels. Tamoxifen and ICI-182 780 blocks E ₂ effects	(Baker et al., 2004)
		DPN	1 nM	Pre-treatment reduces LPS-stimulated iNOS and COX-2 expression	
		PPT	1 nM	No effects	
		ADIOL or E ₂	1 uM	Inhibits the transcriptional activation of inflammatory response genes	(Saijo et al., 2011)
		E ₂	10 & 100 pg /ml	Inhibits LPS-induced elevation of TLR4, p38, and TNF α levels. Also inhibits Kir2.1 channel activity	(Wu et al., 2016)

			1 nM	Blocks the LPS-induced expression of iNOS and restores CD206 to control levels.	(Benedek et al., 2017)
			100 nM	Suppresses pro-inflammatory while enhances anti-inflammatory cytokine gene expression.	(Thakkar et al., 2018)
	Hypoxia	E ₂ or Pg	100 nM	Prevents the induction of iNOS, IL-1 β , and chemokine ligand CCL5; up-regulates IL-10 and TREM 2; prevents the decrease in phagocytic activity induced by hypoxia.	(Habib et al., 2013)
			100 nM	Dampens IL-1 β and the expression of inflammasome components NLRP3 and ASC.	(Slowik et al., 2018)
	β amyloid peptide (A β)	E ₂	10 μ M	Co-treatment reduces expression of iNOS, COX-2 and inhibits NF-kB activation; tamoxifen almost completely reverses E ₂ effects.	(Yun et al., 2018)
	<i>S. pneumoniae</i>	Ginsenoside Rb1	10 μ M	Increases expression of anti-apoptotic proteins Bcl-2 and Bcl-xL and increases phosphorylation of pro-survival signaling molecules Jak2 and STAT5;	(Lee et al., 2018)

				increases expression of ER β and mRNA and protein; stimulates BDNF expression.	
	Apoptotic PC12 cells	E ₂ DPN G1 PPT	100 nM 850 pM 20 nM 200 nM	Enhances phagocytic uptake of apoptotic cells Inhibits phagocytosis activity No effects on phagocytic activity.	(Loiola et al., 2019)
N9 cells	LPS, Phorbol ester and INF- γ	E ₂	0.1 – 10 nM	Pretreatment dose-dependently attenuates microglial superoxide release and phagocytic activity; it also attenuates iNOS protein expression. Effects are blocked by MAPK pathway inhibitor or by IC ₅₀ 182 780	(Bruce-Keller et al., 2000)
	LPS, INF- γ and TNF- α	Estriol, E ₂ , or Pg	50 μ M	Inhibits NO and TNF- α production by activated microglia.	(Drew and Chavis, 2000)

	HIV protein Tat	E ₂	1 nM	Pre-treatment suppresses Tat-mediated microglial activation by interfering with Tat-induced MAPK activation.	(Bruce-Keller et al., 2001)
	LPS	E ₂	1 nM	Increases IL-10 and decreases TNF α and IFN γ release; attenuates LPS-induced MHC Class II, CD40 and CD86.	(Dimayuga et al., 2005)
Primary cultures of <i>neonatal rat</i> <i>microglia</i>					
	LPS	E ₂	1 nM	Blocks LPS-induced increased of nitrite accumulation in media.	(Vegeto et al., 2000)
			1 nM	Blocks microglial morphological changes and the production of inflammatory signals like iNOS, PGE ₂ , MMP-9; effects are blocked by ICI 182 780	(Vegeto et al., 2001)
	LPS, Phorbol ester & INF- γ	E ₂	1 nM	Dose-dependently attenuates microglial superoxide release and phagocytic activity; attenuates of iNOS protein expression. Effects are mediated by MAPK and are blocked by ICI-182 780 treatment.	(Bruce-Keller et al., 2000)

	LPS, INF- γ and TNF- α	Estriol, E ₂ & Pg	50 μ M	Inhibits LPS induction of NO production; repress TNF- α production by activated microglia.	(Drew and Chavis, 2000)
	LPS	E ₂	10 nM	Pre-treatment decreases activation of microglia assessed by morphology, TNF- α mRNA expression, and nitrite production. ICI 182,780 blocks effects, whereas the ER α antagonist MPP-HCL only partially blocks the effects.	(Liu et al., 2005)
		PPT, DPN, E ₂	25 nM 25 nM 100 nM*	Post-treatment prevents cell death and attenuates the release of IL-1 α , IL-1 β , TNF- α , and COX-2.	(Smith et al., 2011)
		E ₂	10 nM	Male neonatal microglia produce greater IL-1 β mRNA than females. Incubation with varying doses of E ₂ produces anti-inflammatory effects in the male microglia but a pro-inflammatory effect in female microglia.	(Loram et al., 2012)

	Hypoxia	E ₂ or Pg	100 nM	Antagonizes hypoxia effects on Trem2 and Arg-1 expression. Lack of effect on hypoxia-induced TNF- α and IL-1 β by E ₂ treatment.	(Habib et al., 2014)
	LPS	Raloxifene & Tamoxifen	0.3, 1 or 3 μ M	Suppress the increase in pro-inflammatory cytokines and chemokines; ICI 182 780 blocks their effects; both SERMS activate ERE-mediate transcription in rat primary microglia.	(Ishihara et al., 2015)
	LPS	E ₂ G1	100 nM 5 nM	Suppress IL-1 β and TNF- α secretion; effects are blocked by the GPR30 antagonist G15.	(Zhao et al., 2016)
Primary cultures of <i>neonatal mouse microglia</i>	LPS	E ₂	1 nM	Prevents the nuclear accumulation of NF-kB via a rapid non-genomic PI3K mediated pathway. Effects are absent in microglia from ER α KO mice.	(Ghisletti et al., 2005)
	OGD/R & LPS	G1 or E ₂	10 nM	Both ligands reverse the OGD/R-induced upregulation of TNF- α , IL-1 β , and IL-6 in both protein extracts and the supernatants and inhibit the up-regulation of TLR-	(Zhang et al., 2018)

				4 protein and mRNA expression. Comparable effects were observed ICI-182 780 plus E ₂ treated cells. G1 reduces LPS-induced NF-κB nuclear expression.	
Primary cultures from <i>adult rat or adult mouse</i> microglia	LPS	E ₂	2 nM	Pretreatment of microglia harvested from olfactory bulbs of OVX young and reproductive senescent rats attenuates the LPS-induced increase in NO.	(Johnson and Sohrabji, 2005)
			10 and 100 nM	Attenuates LPS-induced IL-1β mRNA in female but not male adult hippocampal microglia. E ₂ treatment of females prior to microglia isolation potentiates IL-1β mRNA responses <i>in vitro</i> . LPS-induced decrease in TLR4 mRNA was not affected by sex or E ₂ treatment.	(Loram et al., 2012).
	EAE			<i>In vivo</i> E ₂ treatment of EAE females induces the expression of M2 microglia markers Arg-1 and IL-10 but not CD206 <i>in vitro</i> .	(Benedek et al., 2016).

	Apoptotic PC12 cells	E ₂	100 nM	Promotes phagocytic activity of microglia cells derived from WT but not from adult females carrying a global deletion of the annexin-1.	(Loiola et al., 2019)
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ADIOL, 5-Androstenediol; Arg-1, Arginase 1; ASC, the adaptor molecule apoptosis-associated speck-like protein containing a CARD; Bcl-2, B-cell lymphoma 2; Bcl-xL, B-cell lymphoma-extra-large; BDNF, Brain Derived Neurotrophic Factor; COX-2, Cyclooxygenase 2; CCL5, Chemokine (C-C motif) ligand 5; DPN, diethylpropionitrile; E₂, 17 β -Estradiol; EAE, Experimental autoimmune encephalomyelitis; HIV, human immunodeficiency virus; IL-1 β , Interleukin 1 beta; IL-1 α , Interleukin 1 alpha; IL-6, Interleukin 6; IL-10, Interleukin 10; iNOS, inducible nitric oxide; INF- γ , Interferon gamma; Jak2, Janus kinase 2; LPS, lipopolysaccharide; MMP-9, Matrix metalloproteinase 9; MAPK, mitogen activated protein kinase; NF- κ B, Nuclear factor kappa B; NO, Nitric Oxide; NLRP3, NLR family, pyrin domain-containing 3; OVX, ovariectomy; Pg, progesterone; PGE2, Prostaglandin E2; PI3K, phosphoinositide-3-kinase; PPT, propyl pyrazole triol; STAT5, Signal transducer and activator of transcription 5; TLR4, Toll Like receptor 4; TNF- α , Transforming Growth Factor alpha; TREM 2, Triggering receptor expressed on myeloid cells 2

Table 3. Co-expression of estrogen receptor subtypes in different microglia cell systems.

ER subtype	Model and Sex	Technical approach	References
ERα	Primary microglia cultures from neonatal rat	Double IF and RT-PCR Western Blot and Double IF Single IF and Western Blot	(Vegeto et al., 2001) (Johan and Beyer, 2013) (Ishihara et al., 2015)
	Primary microglia cultures from neonatal mice	RT-PCR, Western blot and double IF Double IF RT-PCR	(Liu et al., 2005) (Elzer et al., 2010) (Smith et al., 2011)
	Primary microglia cultures from adult female mice	FACS	(Loiola et al., 2019)
	N9 microglia cells	Western blot FACS	(Baker et al., 2004) (Dimayuga et al., 2005).
	<i>Ex vivo</i> microglia from adult males (whole brain) <i>Ex vivo</i> microglia from neonatal and adult male and female mice (whole brain)	EGFP-positive microglia sorted by FACS Magnetic bead-assisted FACS followed by RT-PCR	(Sierra et al., 2008) (Crain et al., 2013)

	Brainstem/spinal cord and cortex microglia isolated from young and old female and male mice	Magnetic bead-assisted FACS followed by RT-PCR	(Crain and Watters, 2015)
	Hippocampus of adult male mice	EM	(Sierra et al., 2008)
	Cerebellar microglia from male and female rats	Double IF	(Tapia-Gonzalez et al., 2008)
ERβ	Lumbar SC of male and female mice	Double IF	(Heitzer et al., 2017; Vacca et al., 2016)
	Primary microglia cultures from neonatal rat	RT-PCR RT-PCR, Western blot, Double IF Double IF and Western Blot Single IF, Western blot	(Habibi et al., 2014; Smith et al., 2011; Vegeto et al., 2001) (Liu et al., 2005) (Johann and Beyer, 2013) (Ishihara et al., 2015)
	Primary microglia cultures from neonatal mice	RT-PCR and Western Blot	(Saijo et al., 2011)

	N9 cells	Western blot	(Baker et al., 2004)
		FACS	(Dimas yuga et al., 2005)
	BV-2 cells	Western blot	(Baker et al., 2004; Hidalgo-Lanusa et al., 2018)
		Western blot and RT-PCR	(Saijo et al., 2011)
		RT-PCR	(Habib et al., 2013; Lee et al., 2018)
	Spinal cord female mice	Double IF	(Wu et al., 2013)
GPR30	Lumbar spinal cord male mice	Double IF	(Heitzer et al., 2017)
	Primary murine microglia from adult mice	FACS	(Loiola et al., 2019)
	Motor cortex female rats	Double IF	(Zhao et al., 2016)
	Cortex of female mice	Double IF	(Zhang et al., 2018)

EGFP, enhanced green fluorescence protein; EM, electron microscopy; ER, estrogen receptor; ER α , Estrogen Receptor alpha; ER β , Estrogen Receptor beta; FACS, fluorescence assisted cell sorting; GPR30, G protein coupled receptor 30; IF, immunofluorescence; RT-PCR, reverse transcription, polymerase chain reaction.

