REVIEW

Sex steroid hormones as neuroprotective elements in ischemia models

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Abstract

Among sex steroid hormones, progesterone and estradiol have a wide diversity of physiological activities that target the nervous system. Not only are they carried by the blood stream, but also they are locally synthesized in the brain and for this reason, estradiol and progesterone are considered 'neurosteroids'. The physiological actions of both hormones range from brain development and neurotransmission to aging, illustrating the importance of a deep understanding of their mechanisms of action. In this review, we summarize key roles that estradiol and progesterone play in the brain. As numerous reports have confirmed a substantial neuroprotective role for estradiol in models of neurodegenerative disease, we focus this review on traumatic brain injury and stroke models. We describe updated data from receptor and signaling events triggered by both hormones, with an emphasis on the mechanisms that have been reported as 'rapid' or 'cytoplasmic actions'. Data showing the therapeutic effects of the hormones, used alone or in combination, are also summarized, with a focus on rodent models of middle cerebral artery occlusion (MCAO). Finally, we draw attention to evidence that neuroprotection by both hormones might be due to a combination of 'cytoplasmic' and 'nuclear' signaling.

Key Words

- neuroprotection
- stroke
- brain ischemia
- middle cerebral artery occlusion
- neurosteroids
- estrogens
- progesterone
- neuronal signaling
- glial signaling
- survival response

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Introduction

Estrogens and progestogens are the main female sexual hormones, both mainly synthesized and secreted to the blood by the ovaries (under pituitary control) and placenta and produced by the adrenal glands in both males and females. They have an important function in reproduction; in females, the gonads are the highest contributors to blood levels of progestogens and estrogens before menopause (Konings *et al*. 2017). Among estrogens, estradiol (17-β-estradiol) is considered to be the principal compound with estrogenic action (Hoffman 2006, Perez-Alvarez & Wandosell 2016) whereas progesterone, 5α-dihydroprogesterone and allopregnanolone are considered the main progestogens (Perez-Alvarez & Wandosell 2016).

All estrogens and progestogens are derived from cholesterol; the synthesis pathway first generates pregnenolone, which is afterward converted to progesterone, testosterone and subsequently to estradiol.

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The last step in this pathway, the conversion of androgens to estrogens, is catalyzed by aromatase, which is expressed in different tissues including the brain (Azcoitia *et al.* 2017). The tissue availability of estrogens and progestogens depends on circulating levels of both hormones, but is also determined by local, *de novo* synthesis in various non-reproductive tissues, including the brain (Guennoun *et al.* 2015, Konings *et al.* 2017). Indeed some of the enzymes responsible for the synthesis of estradiol have been described in neurons and glial cells (Pelletier 2010).

Due the fact that some brain cells have the capacity to locally synthesize them, estrogens and progestogens are not only 'neuroactive steroid' compounds, but can be considered 'neurosteroids'.

Physiological action of estradiol in the brain

In addition to reproductive functions (Sinchak & Wagner 2012), estrogens can modulate important brain functions in males and females including cognition, learning and memory, mood, pain and nociception (Roselli 2007). These physiological effects are due to the ability of estradiol to generate substantial changes in neuronal physiology and morphology of key brain areas such as the hippocampus, striatum, amygdala, cortex and hypothalamus, among others (Carrer & Aoki 1982, Korol & Pisani 2015).

It is known that all the cellular components of the central nervous system (neurons, glial cells and endothelial cells) are targets of estradiol, which regulates neuronal and glial cell differentiation in various brain areas like the hypothalamus (Díaz *et al.* 1992, Lenz & McCarthy 2010), midbrain (Beyer & Karolczak 2000), cerebellum (Tsutsui 2006, 2012), hippocampus (Bender *et al.* 2010) and neocortex (Hu *et al.* 2007), among others.

In addition, numerous reports have confirmed a substantial neuroprotective role for estradiol in models of neurodegenerative diseases, but the focus of this review is on traumatic brain injury and stroke models (Pérez-Alvarez & Wandosell 2013, Engler-Chiurazzi *et al.* 2016, Melcangi *et al.* 2016, Perez-Alvarez & Wandosell 2016).

Most of the studies involving estrogenic actions in the brain have focused on neurons. Many studies indicate that estradiol controls important mechanisms that regulate neuronal maturation and differentiation, such as axonal and dendritic development, growth of dendritic spines and the establishment of synapses (Arevalo *et al.* 2012). However, the underlying molecular mechanism implicated in each of these developmental activities of estradiol is not necessarily identical; it is possible that the mechanism depends on neuronal type and/or brain region, and specific repertoires of hormone receptors. Also, since estradiol can be synthesized by neurons and glia, these actions may be mediated by paracrine or autocrine mechanisms (Kretz *et al.* 2004, Von Schassen *et al.* 2006, Hu *et al.* 2007, Fester *et al.* 2009). It has been established that brain levels of aromatase are a reliable indicator of the amount of brain-derived estradiol. Under physiological conditions in mammals, amphibians, reptiles and birds, aromatase is located mainly in neurons (Coumailleau *et al.* 2014). However, in teleost fish, aromatase has been detected principally in radial glia (Forlano *et al.* 2001).

Estradiol has an important impact on brain development and normal physiology, but also after injury. Cellular targets of its action are both neurons and the main glial cell types (astrocytes, microglia, radial glia and oligodendrocytes) (Pérez-Álvarez *et al.* 2012, Pérez-Alvarez & Wandosell 2013). After brain injury, ischemia or hypoxia, levels of aromatase (and consequently estradiol) increase in the damaged area, especially in astrocytes and radial glia, but also in neurons (Garcia-Segura *et al.* 1999, Peterson *et al.* 2001). Nowadays, this neurosteroid is considered to have an important role in neuroprotection, acting locally through a rapid mechanism of action (see below) (Garcia-Segura *et al.* 1999, Pedersen *et al.* 2016). One of the estradiol-mediated neuroprotective mechanisms is the significant reduction of neuroinflammation, a common effect after brain trauma or injury that exacerbates initial damage (reviewed in Perez-Alvarez & Wandosell 2016). Several studies have demonstrated in mammals and birds that the anti-inflammatory effect of estradiol plays a protective role, positive when local estradiol production in astrocytes is increased after upregulation of aromatase expression or negative when its activity is inhibited (Garcia-Segura *et al.* 1999, Pedersen *et al.* 2016).

Estradiol receptors: 'classical' and 'non-classical' pathways

Estrogens initiate biological actions by binding specific proteins named estradiol receptors (ERs), which are classified into two main groups. The first, known as the 'classical' ERs (ERα and ERβ), belong to the nuclear receptor superfamily and act as nuclear transcription factors (Hewitt *et al.* 2010). The second group, 'non-classical' ERs (G-protein-coupled estradiol receptors), are membrane-associated receptors responsible for rapid and non-genomic estrogenic action, including GPER1

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(also known as GPR30) and Gq-mER. Although GPER1 is a transmembrane receptor, it has also been reported on intracellular compartments, such as the endoplasmic reticulum (Revankar *et al*. 2005, Thomas *et al*. 2005, Filardo *et al*. 2007, Alexander & Harvey 2017), whereas Gq-mER was originally identified in hypothalamic neurons. Their function is poorly understood, but seems to have a modulatory role in HPA axis (Qiu *et al*. 2008, Nag & Mokha 2014, Hu *et al*. 2016).

'Classical' ERs have been detected in the extranuclear compartment; in fact, ERα was identified on the plasma membrane of neurons and astrocytes (Dominguez & Micevych 2010, Azcoitia *et al*. 2011). ERα can also interact with other membrane receptors, generating macromolecular complexes that allow for synergy with other growth factors. Regarding the cell types expressing ERs, our research group and others have reported that both neurons and glial cells express ERα and ERβ (Varea *et al*. 2010). The expression of GPR30 in neurons and astrocytes has been demonstrated using pharmacological approaches (Lee *et al*. 2012, Tang *et al*. 2014), though the presence of Gq-mER in glial cells has not been fully demonstrated. Many reports have indicated that, at least in the mammalian brain, neurons and glia respond specifically to estradiol through ERs, even though the exact contribution of ERα, ERβ, GPR30 and Gq-mER in each neural cell type has not been established (Shughrue *et al*. 1997, Toran-Allerand *et al*. 1999, Hübner *et al*. 2015).

Similar to ER classification, the estrogenic mechanisms of action in target tissues are sorted into 'classical' (transcription-dependent) and 'non-classical' (cytoplasmic-dependent, receptor-independent antioxidant actions) (Mann *et al*. 2007). 'Classical' pathways are nuclear actions regulated by 'classical ERs', in which the main effect is to regulate gene expression at a transcriptional level. The interaction of estrogens and ERs forms a homodimeric or heterodimeric complex that binds to specific DNA sequences called estrogen response elements (ERE), activating gene expression (Pettersson *et al*. 1997). Estrogen-ER complexes can also interact with other nuclear receptor coactivators or repressors (Gruber *et al*. 2002) (summarized in Fig. 1). Usually, slow effects with long-term actions, typical of a steroidal hormone response, characterize this pathway.

The 'non-classical' pathway is characterized by rapid effects mediated by membrane-associated ERs, with a critical role in the nervous system and neuroprotection (Toran-Allerand *et al*. 2002, Raz *et al*. 2008). In fact, estradiol may activate different signaling pathways such as MAPK/Erk (Singh *et al*. 1999), PI3K/Akt (Honda *et al*. 2000), PKA (Prossnitz & Barton 2011) or PKC (Cordey *et al*. 2003), thus regulating multiples genes and neuronal functions including apoptosis/survival. Some of these actions may be attributed to the G-protein-coupled receptor (GPR30), but not all (Filardo & Thomas 2005, Prossnitz *et al*. 2007, 2008) (summarized in Fig. 1). In addition, estrogens have been reported to present receptor-independent antioxidant actions (Pajović & Saičić 2008).

Our group has demonstrated that ERα interacts with the PI3K regulatory subunit p85 in the brain and primary neuron cultures, coupled in this way to the 'cytoplasmic signaling' of insulin-like growth factor 1 receptor (IGF1R). The interaction between ERα and p85 is associated with GSK3 phosphorylation and β-catenin stabilization (Cardona-Gomez *et al*. 2004). This macromolecular complex in neurons may contain ERα, IGF1R and some components of IGF1R downstream signaling such as phosphoinositide 3-kinase (PI3K), AKT, glycogen synthase kinase 3β (GSK3β) and β-catenin. The complex is sensitive to IGF-1 and estradiol, with effects in neurons and glia that allow synergistic action between them (Mendez *et al*. 2003, Alonso *et al*. 2008, Garcia-Segura *et al*. 2010, Arevalo *et al*. 2012).

As mentioned earlier, estradiol activates β-catenin-mediated transcription through the PI3K–AKT–GSK3 signaling pathway (Varea *et al*. 2009, 2010, 2013). Inhibition of GSK3β activity is a common mechanism of neuroprotection initiated by several factors, such as Wnt, IGF-1, neurotrophins and estradiol. In addition, it has been reported that estradiol activates β-catenin-mediated transcription through the canonical WNT signaling pathway, which is activated when WNT binds its co-receptors (low-density lipoprotein-related protein 5 (LRP5), LRP6 and Frizzled), and negatively regulated by Dickkopf 1 (DKK1). Some reports have indicated that estradiol upregulates WNT signaling by inhibiting expression of DKK1 (Zhang *et al*. 2008, Scott *et al*. 2012). Whether both estradiol effects (via GSK3 phosphorylation/β-catenin stabilization and DKK-1-derived effects) are sequential events or not must be more deeply analyzed (some of these data are schematically represented in Fig. 1).

Physiological action of progesterone in the brain

As mentioned earlier, progestogens are neurosteroids that are generated locally in the nervous system, as different areas of the brain and spinal cord express the enzymes

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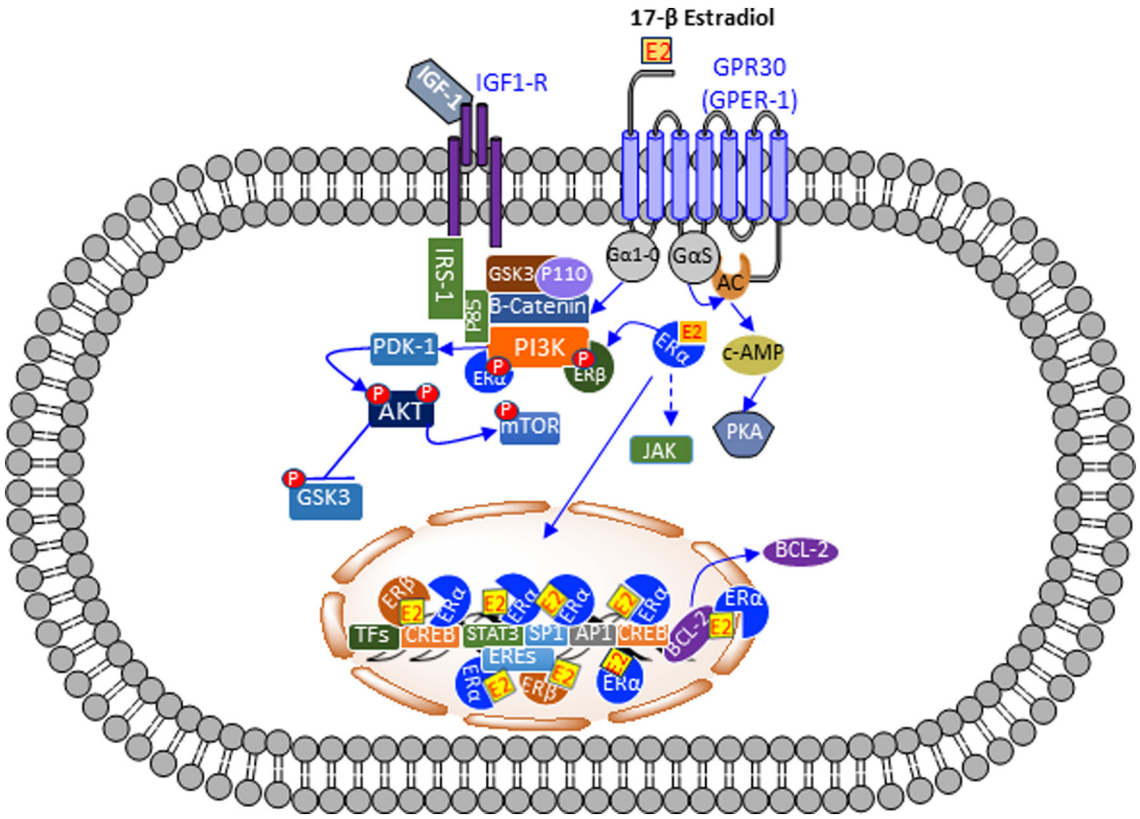

Figure 1
Schematic representation of classical and non-classical pathways of estrogen receptors (ERs). 17-β-estradiol is represented as E2. The 'classical receptors', ERα and ERβ, after linking E2, form complexes that act as transcription factors by direct binding with specific DNA regions (EREs) (either homo or heterodimeric) or by binding to some co-transcriptional activators (CREB, STAT, SP1, etc.), dependent on different signals: Expression and activation of multiple targets, such as BCL-2, may play a key role in neuroprotective actions, although the complete molecular mechanisms remain to be elucidated. Some elements of the IGF1/IGFR pathway were represented. These classical receptors (ERα and/or ERβ) may form a complex with the regulatory element of PI3K class I, p85 in conjunction with GSK3/β-catenin. The binding of E2 to ERs in this complex finally stabilizes β-catenin, which may act as a co-transcriptional factor in the nucleus, similar to Wnt signaling. Besides the membrane-bound estrogen receptor GPR30 trigger a cytoplasmic signaling through the activation of Gi and/or Gs proteins, acting on PKA, PI3K or JAK kinases among others. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0129.

required for progesterone and allopregnanolone synthesis (Do Rego *et al*. 2012, Melcangi *et al*. 2014, Schumacher *et al*. 2014, Guennoun *et al*. 2015), in both neurons and glial cells (Guennoun *et al*. 2015).

In neurons, progesterone regulates differentiation, synaptic plasticity, dendritic remodeling and neurogenesis (Woolley & McEwen 1993, Giachino *et al*. 2003, Foy *et al*. 2008). In glial cells, it contributes to differentiation and proliferation of astrocytes (Luquin *et al*. 1993, Jung-Testas *et al*. 1999, Arbo *et al*. 2016), oligodendrocytes (Ghoumari *et al*. 2005), and microglia (Roche *et al*. 2016) and plays a key role in myelinogenesis (Deutsch *et al*. 2013). It should be noted, that some of the effects observed after progesterone administration may be triggered by their derivative, allopregnanolone (for a review see: Noorbakhsh *et al*. 2014, Guennoun *et al*. 2015).

In addition, progesterone plays a role in neuroprotection after stroke, reduces neuroinflammation and decreases *reactive gliosis*, among other effects (Brinton *et al*. 2008, Deutsch *et al*. 2013, Arbo *et al*. 2016, Perez-Alvarez & Wandosell 2016).

Progesterone and its receptors

The biological actions of progestogens are initiated by their binding to specific receptors, all of them expressed in the central nervous system (Guennoun *et al*. 2015). Similar to estradiol, progesterone receptors can be grouped into two main families: the 'classical' progesterone receptors (PRs), PR-A and PR-B, that belong to the nuclear receptor superfamily of transcription factors (Kastner *et al*. 1990); and membrane-associated receptors (mPR and the

PGRMC1 complex) that can stimulate rapid intracellular signaling. In addition, modulation of GABA-A receptor activity by allopregnanolone has a direct impact on neuronal activity, related to neuroprotection and pain modulation (Sitruk-Ware & El-Etr 2013, Afrazi & Esmaeili-Mahani 2014, Schumacher *et al.* 2014, Huang *et al.* 2016). A single gene that differentially expresses several subdomains encodes classical PRs, including a variable N-terminal region, a conserved DNA-binding domain, a variable hinge region, and a conserved ligand-binding domain. PR-B, the longer isoform, contains an additional N-terminal region that is missing in PR-A, known as the B-upstream segment (BUS), which represents an additional activation region (Conneely *et al.* 1987, Diaz Brinton 2012). Some short isoforms (PR-A) have been described lacking the DNA-binding domain and nuclear localization signal (NLS) (Hirata *et al.* 2002); it has been proposed that these shorter versions may function as cytoplasmic signaling elements, alone or coupled with membrane-associated PRs (see below).

Regarding mPRs, five isoforms have been described to-date: mPRα, mPRβ, mPRγ, mPRδ and mPRε (Zhu *et al.* 2003*a*, Pang *et al.* 2013), all of which are 7-transmembrane proteins that activate G proteins. They are members of the progestin and adipoQ receptor (PAQR) family, rather than members of the G protein-coupled receptor superfamily (Thomas & Pang 2012). All mPR mRNAs are expressed in the central nervous system at variable levels (Zuloaga *et al.* 2012, Meffre *et al.* 2013, Pang *et al.* 2013, Schumacher *et al.* 2014). mPRα has been found in neurons and is strongly induced in some glial cell (astrocytes, oligodendrocytes and microglia) after local injury, indicating a potential modulatory role in neuroinflammation (Meffre *et al.* 2013, Guennoun *et al.* 2015).

PGRMC1 is another less-known component of membrane PRs, mostly expressed in the spinal cord, hypothalamus, ependymal cells, circumventricular organs and meninges (Meyer *et al.* 1996, Meffre *et al.* 2005, Labombarda *et al.* 2013, Guennoun *et al.* 2015). After local injury, PGRMC1 expression increases in neurons and astrocytes (Meffre *et al.* 2005, Guennoun *et al.* 2008).

Mechanism of action of progesterone

Based on the two described groups of PRs, and similar to estrogens, the mechanisms of progesterone action can be divided into 'classical' and 'non-classical'. The 'classical' mechanism is triggered by PRs and is characterized by its slow action: progesterone binding to a PR induces receptor dissociation from chaperones, homo- or hetero-dimerization, and finally activation of gene transcription. This activity is mediated by direct binding to progesterone response elements (PREs) in the promoter region of target genes (Schumacher *et al.* 2014). Current data emphasizes the importance of recruitment of coactivators like SRC1, SRC2 and SRC3, among others. The transcriptional activities of nuclear receptors are modulated in a positive or negative manner, depending on the availability of coactivators or corepressors (York & O'Malley 2010, Amazit *et al.* 2011, Schumacher *et al.* 2014). This increases the complexity of understanding the mechanisms of progesterone, because the response is not only dependent on progesterone and PRs, but also coactivators/corepressors that have variable expression and distribution throughout the brain. In addition, the supplementary relationship between PR-A and PR-B increases the complexity of this transcriptional system (Brayman *et al.* 2006, Aupperlee & Haslam 2007).

Both receptor groups, PRs and mPRs initiate the 'non-genomic or rapid' actions of progesterone. PRs are able to activate kinases such as MAPK, PI3K, CDK and c-SRC in a direct manner (by association with cytosolic kinases) or indirectly through binding to membrane-associated complexes (Migliaccio *et al.* 1998, Boonyaratanakornkit *et al.* 2001, Schumacher *et al.* 2014). This extranuclear signaling of PRs may play a key role in neurons, as some evidence shows PR localization in axons, dendrites and synapses (far from the nucleus), highlighting the possible role of PRs in neuronal activity and excitability (Schumacher *et al.* 2014). The five mPR types allow for differential signaling, the best known being direct interaction with protein Gs or Gi, resulting in activation (in mPRα, mPRβ and mPRγ) or inhibition (in mPRδ and mPRε) of the MAPK pathway through modulation of cAMP production (Zhu *et al.* 2003*b*, Thomas 2008). mPRα (via MAPK activation) has been implicated in the anti-apoptotic effects of progesterone (Pang *et al.* 2013). Knowledge about the mechanism of action of PGRMC1 is poor, though a recent report related PGRMC1 activation by progesterone to neuroprotection through the release of BDNF by astrocytes (Su *et al.* 2012) (summarized in Fig. 2).

Obviously, there exists some cross-talk between extranuclear and classical intracellular signaling pathways, which must be taken into account to understand the full mechanism of action of progesterone (Mani & Oyola 2012). It is also very important to remember the close relationship between progesterone and allopregnanolone, which is a potent activator of the GABA-A receptor, enhancing the inhibitory transmission of GABA with

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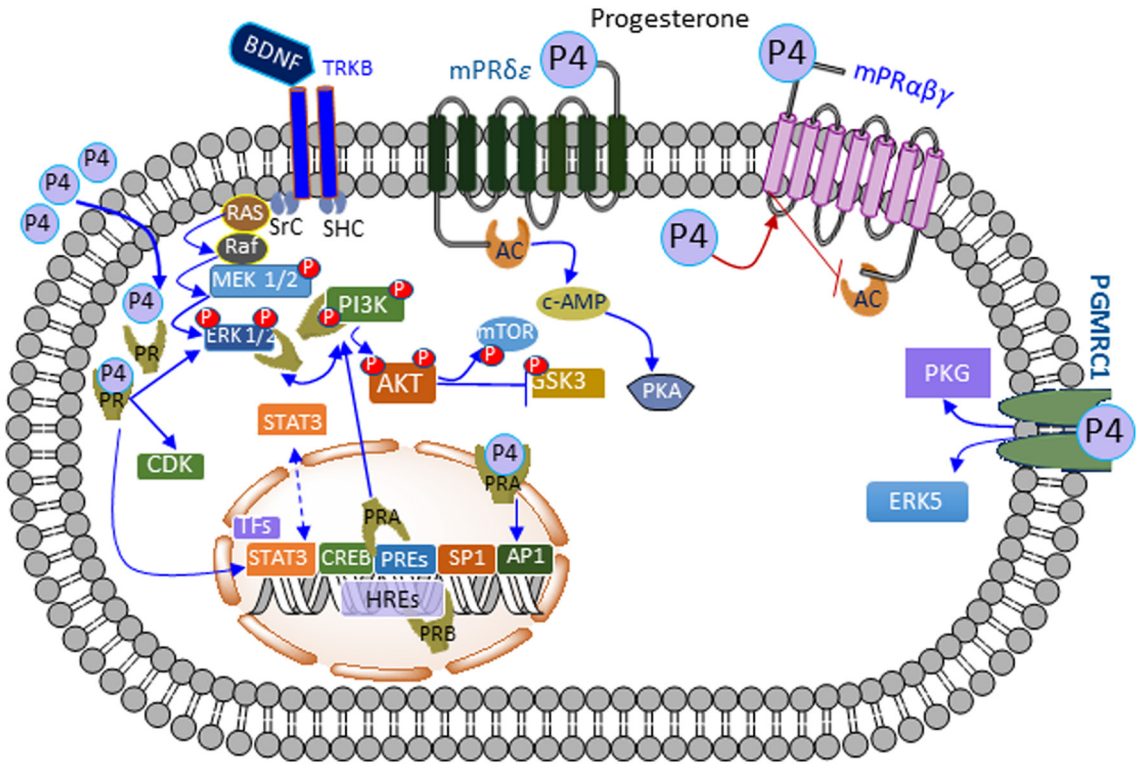

Figure 2
Schematic representation of classical and non-classical pathways mediated by progesterone receptors (PRs). Progesterone is represented as (P4). The 'classical' progesterone receptors (PRs) PR-A and PR-B belong to the superfamily of nuclear receptors and that activate genes for transcriptional regulation, by direct binding to specific DNA regions (HREs) or bind to co-transcriptional activators (such as, CREB, STAT, SP1, etc.). Some data indicated that PR A and/or B may triggered or boost MAP kinase signaling, directly or indirectly. Besides this nuclear transcriptional regulation, progesterone receptors located on the plasma membrane (mPRs) that activate intracellular signal have been described. Accordingly mPRδ and mPRε may activate PKA, via adenylate cyclase (AC), whereas mPRα/β/γ may inactivate AC. In addition a different membrane-associated receptor denoted as PGMRC1 have been reported and may act as a co-receptor to trigger rapid intracellular signals (such as Erk5 or PKG). A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0129.

additional physiological consequences (Belelli *et al*. 2006, Hosie *et al*. 2006).

Therapeutic effects of estradiol and progesterone: synergists or antagonists?

In general, neurosteroids (particularly progesterone and estradiol) have been considered neuroprotective, shown by various animal models of neurodegenerative diseases, stroke, spinal cord damage and traumatic brain injury (Perez-Alvarez & Wandosell 2016, Schumacher *et al*. 2016). However, for several reasons, this idea does not always correspond well to the therapeutic effect observed in patients, so the use of estradiol and progesterone in humans remains a controversial subject.

In fact, the reduction of sex hormones in women during normal aging has been associated with the brain's vulnerability to some diseases (Merchenthaler *et al*. 2003), supporting that female sex hormones are neuroprotective agents. Indeed, there is a lot of evidence in the human population to consider estradiol and progesterone as neuroprotective. The normal age-related decline of female sex hormones during menopause is strongly related to greater incidence of certain diseases including neurodegenerative and cardiovascular disorders and stroke. The onset and severity of neurodegenerative disease has a marked difference between the sexes, being especially evident at menopause. In fact, menopause is considered a risk factor for stroke (Wise 2003) among other neurodegenerative disorders (Xu *et al*. 1998, Simpkins *et al*. 2005, Bourque *et al*. 2009, Schreihofer & Ma 2013). Although the circulating levels of both estradiol and progesterone progressively decrease during menopause, the neuroprotective effect is mainly attributed to estradiol, probably due to the fact that blood-estradiol levels in adult women are higher than progesterone.

This decline in neuroprotection may have several reasons, not only due to the reduction in circulating

estradiol; it has been reported that estradiol receptors (ERα and ERβ) present age- and menopause-related differences. Thus, increasing age was associated with decreasing ERα and ERβ expression (Park *et al*. 2017). Indeed, it is well accepted that ERα expression is autoregulated by estradiol, which may explain a lower level of receptors in some brain regions, associated with age and with some pathologies such as Alzheimer's disease (AD) (Wijayaratne & McDonnell 2001, Mott & Pak 2013, Wang *et al*. 2016).

Thus, it is tantalizing to propose that even considering the capacity of astrocytes to overexpress aromatase, the reduction of ERs may generate a lower sensitivity to response during aging and menopause. Thus, diminishing levels of circulating estrogen in postmenopausal women, coupled with a reduction of brain estrogen receptors, may contribute to the inflammatory state that is a hallmark of many neuropathologies (Yague *et al*. 2006, Luchetti *et al*. 2011, Medway *et al*. 2014).

Further, *in vitro* and *in vivo* models have demonstrated the beneficial effects of estradiol administration pre- or post-injury for some diseases including glutamate excitotoxicity (Hilton *et al*. 2006), stroke (Pérez-Álvarez *et al*. 2012), neuroinflammation (Perez-Alvarez & Wandosell 2016) or AD (Xu *et al*. 1998). It is interesting to note that some of the beneficial effects of estradiol after brain injury can be attributed to direct anti-inflammatory effects on glial cells after local synthesis (Bruce-Keller *et al*. 2000, Baker *et al*. 2004, Vegeto *et al*. 2006, 2008, Pérez-Álvarez *et al*. 2012) (Pedersen *et al*. 2016).

Similar to estradiol, progesterone is considered a neuroprotective mediator (De Nicola *et al*. 2006, Sayeed & Stein 2009, Guennoun *et al*. 2015), with positive effects after damage including reduction of inflammation after traumatic brain injury (Pettus *et al*. 2005), decreased oxidative damage (Roof *et al*. 1997), protection of neurons from excitotoxicity (Smith 1991) and recovery of mitochondrial dysfunction (Irwin *et al*. 2008). In addition, there are many similarities between the actions of progesterone and estrogen, such as their signals and targets. In spite of this, the underlying mechanism of action of estradiol- or progesterone-mediated neuroprotection remains unclear. There are surprisingly few studies and clinical trials using a combination of estradiol and progesterone, and they have shown mixed results.

Some experiments revealed a synergistic or additive effect of combined administration of estradiol and progesterone compared with estradiol alone, such as preventing cell death via diminution of apoptosis (Nilsen & Brinton 2002*b*) or improving myelination in autoimmune encephalomyelitis (Garay *et al*. 2008). However, other reports indicated a non-synergistic or even antagonistic effect, seemingly contradictory results which may be reasonable because in almost all cases of combination treatment, the positive response reported for estradiol were reduced or almost blocked by progesterone (Aguirre & Baudry 2009, Lorenz *et al*. 2009, Yao *et al*. 2011, Maki & Henderson 2012, Perez-Alvarez *et al*. 2015). In long-term experiments, it is important to remember that progesterone modulates the availability of ERs (Aguirre *et al*. 2010) and that estradiol regulates the differential expression of PRs (Acharya *et al*. 2015). Moreover, it is well known that prolonged treatment with estradiol increases the risk of some hormone-dependent cancers, and in these cases, the combination with progesterone may be critical to reduce undesirable estrogenic side effects. Another factor to consider is the time of action, perhaps using short-term vs long-term treatments to enhance synergistic neuroprotective effects, as has been suggested for AD in women (Wharton *et al*. 2011, Shao *et al*. 2012).

Estradiol and progesterone: therapeutic effects in stroke models

Ischemic stroke is a complex neurodegenerative disease with high global prevalence, being the second-leading cause of death and the leading cause of disability (Mozaffarian *et al*. 2016). Human epidemiological data show a marked gender difference related to incidence and prognosis of stroke: Men have higher incidence and worse prognosis for stroke than women do until menopause, at which point the pattern is reversed (Scott *et al*. 2012, Mozaffarian *et al*. 2016). Therefore, statistical data suggest that sex hormones play a pivotal role in brain damage after stroke, and studies in animal models sustain this notion. For instance, in age-matched rats, females had less serious brain injury than males after induction of cerebral ischemia, while ovariectomized females presented more brain damage than intact females or even males (Alkayed *et al*. 1998*b*, Rusa *et al*. 1999, Inagaki & Etgen 2013).

The pathophysiological events triggered after cerebral ischemia, termed 'ischemic cascade', have been determined using animals models. Middle cerebral artery occlusion (MCAO) is one of the most extensive *in vivo* models used for research, and may be transient (tMCAO) or permanent (pMCAO), depending on the duration of occlusion (Sawada *et al*. 2000). The ischemic cascade is composed of highly time-dependent phases including energetic failure, excitotoxicity, peri-infarct depolarizations, neuro-inflammation and cell death that

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may be necrotic or programmed (apoptosis) (Dirnagl *et al.* 1999; Pérez-Alvarez & Wandosell 2013) (summarized in Fig. 3). Neuroprotective agents against ischemia block or reduce some aspects of this ischemic cascade. There is evidence that some of the pathological actions of the ischemic cascade are modulated by estradiol and/or progesterone (Perez-Alvarez *et al.* 2015). However, although more than 1026 potential neuroprotective drugs have been tested in animals with promising results, at present none have been successful in humans (Jickling & Sharp 2015).

The neuroprotective effects of estradiol administration alone have been widely described in stroke models (Sawada *et al.* 2000). Blood-estradiol levels are inversely correlated with infarct size after ischemia induction (Liao *et al.* 2001), and the described positive effects of estradiol also occur it is exogenously administered before or after ischemia, in both males and females (Alkayed *et al.* 1998*a*, Dubal *et al.* 1998, Toung *et al.* 1998, Pérez-Álvarez *et al.* 2012). As indicated earlier, brain-estradiol levels are very dependent on local synthesis by aromatases, and after ischemic injury, the expression of aromatase increases mainly in astrocytes (McCullough *et al.* 2003, Carswell *et al.* 2005, Scott *et al.* 2012, Pedersen *et al.* 2016).

One of the beneficial actions of estradiol in the ischemic cascade is modulation of the neuroinflammation

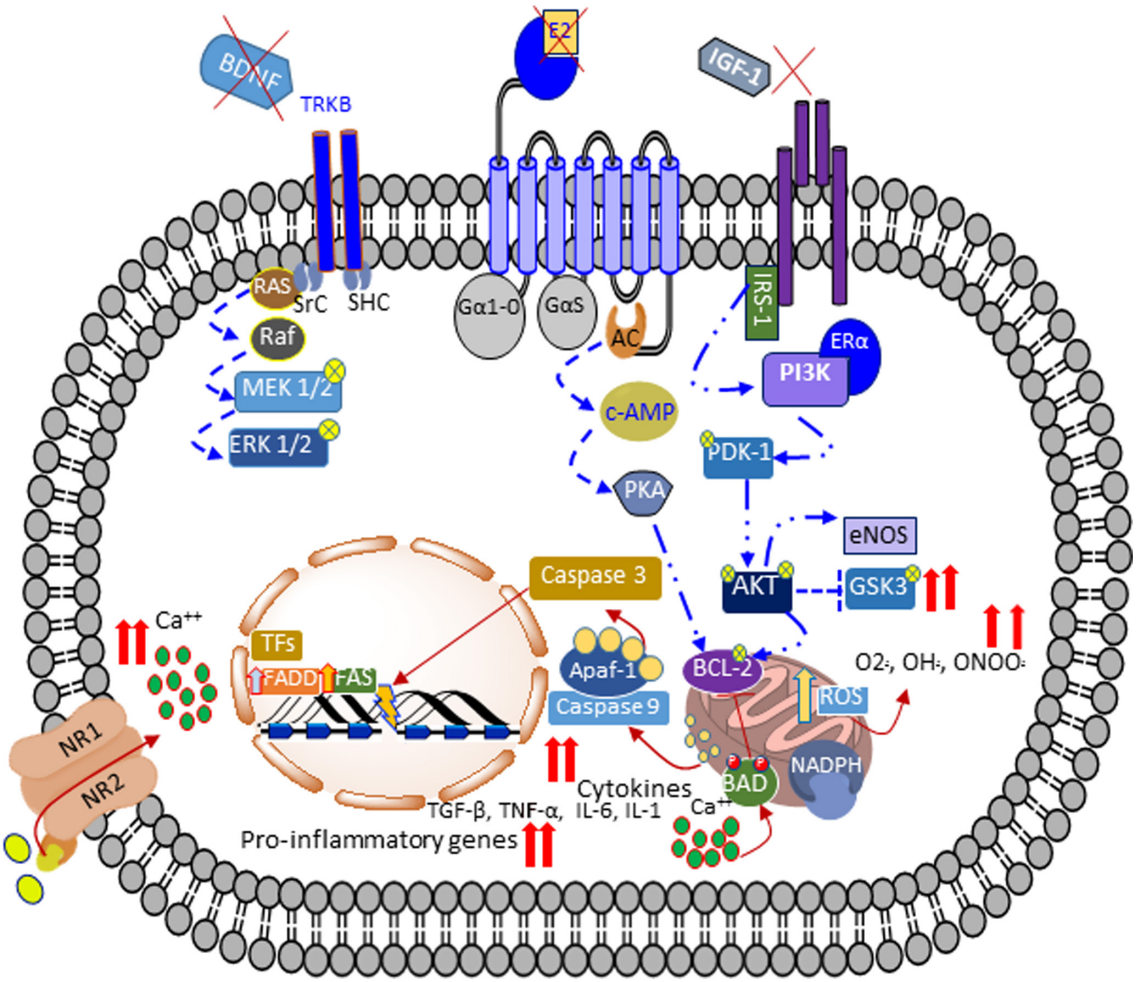

Figure 3

The ischemic cascade may have effect on diverse molecules that participate in neuronal function. The ischemia impedes the supply of several nutrients to neurons, among them hormones and neurotrophins. Thus, the reduction of IGF1/insulin, E2 or neurotrophins, such as BDNF will reduce the signals trough the corresponding receptors, such as IGFR or TrkB). This inactivation/downregulation (represented as dotted lines) will reduce the signals of several pathways such as of PI3K-AKT, generating an imbalance survival/apoptosis. Consequently, dephosphorylation and inactivation of the protein such as, anti-apoptotic BCL-2 or phosphorylation of pro-apoptotic BAD protein, and generation of free radical, and ROS and cytochrome C release activates caspases 3 and 9, leading to DNA rupture and cell death. In addition, high levels of glutamate lead to an over activation of ionotropic and metabotropic glutamate receptors, with a massive influx of sodium and calcium that cause excitotoxicity and cellular death (red arrows represent the pro-apoptotic elements augmented or over-activated). A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0129.

response. It is well known that local activation of microglia and astrocytes, known as reactive gliosis, is accompanied by the synthesis and release of several chemokines and cytokines (Dhandapani *et al*. 2005, Arevalo *et al*. 2013, Perez-Alvarez & Wandosell 2016).

Our group and others have reported that post-ischemic estradiol treatment reduces reactive gliosis (Pérez-Álvarez *et al*. 2012) and attenuates the production of some pro-inflammatory cytokines after ischemia through activation of ERs (Chiappetta *et al*. 2007, Suzuki *et al*. 2007), in parallel with a size reduction of the injured brain region. Since estradiol acts simultaneously on glia and neurons, there is no clear picture of how estradiol impacts the different players involved in ischemic progression (neurons, glia and endothelial cells), nor the implications of this for the pathophysiological events associated with ischemic brain injury (summarized in Fig. 4).

From a molecular point of view, there is a large body of evidence supporting a role for estradiol in the rapid activation of neuronal signaling pathways related to neuronal survival, such as PI3K-AKT-GSK3 (De Butte-Smith *et al*. 2012, Perez-Alvarez *et al*. 2012) and ERK/MAPK (Jover-Mengual *et al*. 2007, Koh 2007), among others (Sawe *et al*. 2008). In many neurotoxic models, enhancement of these pathways may prevent neuronal death (Pap & Cooper 1998, Parcellier *et al*. 2008), though to-date the question about which receptor subtype plays a predominant role in estradiol-mediated neuroprotection from stroke has not been resolved. Experimental evidence *in vivo* suggests that ERβ does not play an essential role in neuroprotection after stroke, as administration of an ERβ-selective agonist (diarylpropionitrile, DNP) was not significantly protective in a stroke model (Farr *et al*. 2007, Connell & Saleh 2011). Studies in ERβ-knockout mice showed that exogenous administration of estradiol continued to protect the brain against ischemia (Dubal *et al*. 2001, 2006). In contrast, ERβ was shown to induce neuroprotection *in vitro* (using an excitotoxic model of hippocampal neurons) (Zhao & Brinton 2007), whereas a selective agonist of GPR30 (G-1) has been reported to exert neuroprotection against cerebral ischemia in tMCAO (Zhang *et al*. 2010). Considering all the contradictory

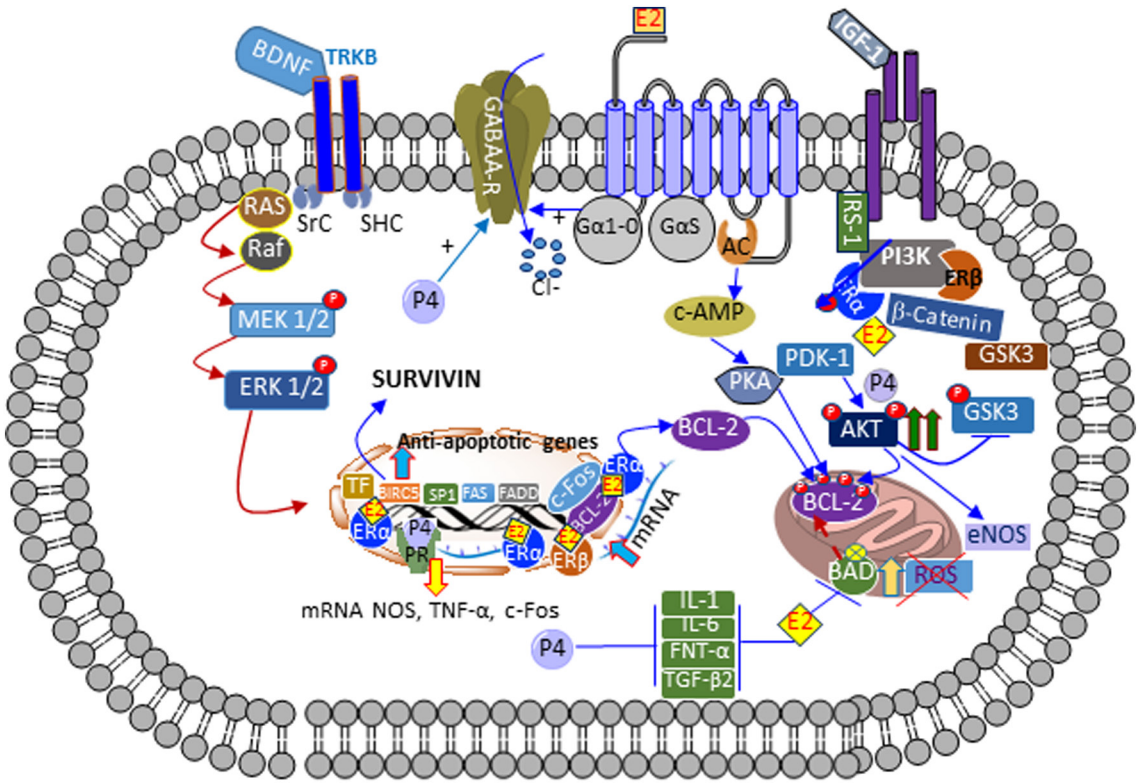

Figure 4
Administration of estradiol and progesterone may reduce ischemic damage. The pharmacological application of E2/P4, in combination, may restart some 'rapid' cell signaling pathways from both estrogen and progesterone, either by membrane receptors or by intracellular counterparts. The activation of E2 and/or P4 signaling may enhancement the PI3K-AKT and MAPK pathway. Besides, both E2 and P4 signaling reduce expression of pro-apoptotic genes and levels of pro-inflammatory cytokines, modulating activity of ionotropic (GABA-A, glutamate) receptors, promoting survival. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0129.

results, we can conclude that more work is needed to be able to identify the specific ERs responsible for the neuroprotective action of estradiol.

Information about the neuroprotective effects of progesterone after ischemic damage is not as extensive as for estradiol. It is generally accepted that progesterone attenuates the brain damage induced by ischemia, with some reports showing protective effects in animal models (Roof *et al*. 1997). Progestogens can act directly in neurons or by modulating glial activation (Perez-Alvarez & Wandosell 2016), and some results indicate that progesterone reduces ischemic damage, acting at different levels of the ischemic cascade. Progesterone ameliorates neuronal excitotoxicity, preventing increase of cytosolic calcium by blocking calcium channels and upregulation of GABA-A receptors (Smith 1991, Limmroth *et al*. 1996, Shen *et al*. 2005) and decreases neuronal apoptosis by inhibition of caspase-3 activity and upregulation of anti-apoptotic proteins such as Bcl-2 (Nilsen & Brinton 2002*a*, Anderson *et al*. 2011). Moreover, progesterone may reduce vasogenic and cytotoxic edema.

Progesterone has been shown to be anti-inflammatory, reducing neuroinflammation by different mechanisms (for a review see: Perez-Alvarez & Wandosell 2016). Progesterone modulates cytokine release, significantly suppressing ischemia-induced upregulation of IL-1β and TGF-β2 mRNAs in the ischemic area and also reduces levels of pro-inflammatory cytokine mRNAs (Gibson *et al*. 2005, Chiappetta *et al*. 2007). In addition, it reduces peripheral immune cell activation and migration and microglia activation (Roof & Stein 1992, Drew & Chavis 2000, Wright *et al*. 2001, He *et al*. 2004, Pettus *et al*. 2005, VanLandingham *et al*. 2007). All of this anti-inflammatory progesterone activity goes in parallel with a decrease in lesion volume and a reduction of ischemic damage (summarized in Fig. 5).

Experimental data about combined use of estradiol and progesterone for *in vivo* or *in vitro* models of stroke are scarce and contradictory (Roof & Hall 2000), with some pointing to synergistic action and others to non-additive effects (Dang *et al*. 2011, Perez-Alvarez *et al*. 2015). Our studies have shown that the combination of estradiol/progesterone in an *in vivo* (pMCAO) or *in vitro* oxygen-glucose deprivation (OGD) model reduced some protective effects of estradiol in the cerebral cortex but not in the hippocampus (Perez-Alvarez *et al*. 2015), revealing that it is brain region dependent. In fact, estradiol administration after pMCAO greatly reduced reactive gliosis and led to recovery of the PI3K/AKT/GSK3 pathway that had been downregulated by ischemia, whereas the

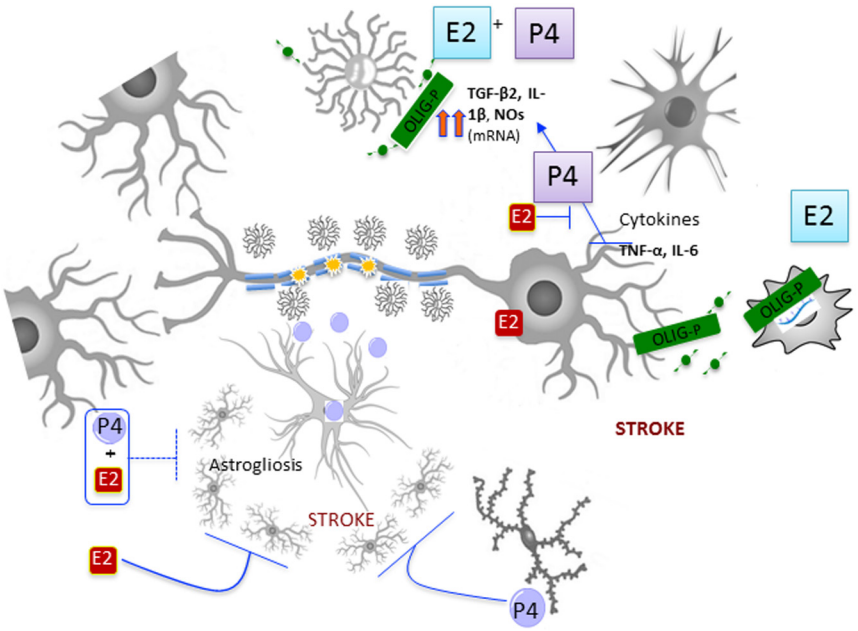

Figure 5
Interaction between neurons, astrocytes and microglial cells during brain injury and the involvement of progesterone and estradiol in regulation and restoration. Stroke induced general activation of glia known as reactive gliosis. These cellular events induce local synthesis of neurosteroids, an endogenous mechanism of neuroprotection. Progesterone can be synthesized in both neurons and quiescent (non-reactive) astrocytes, while E2 is synthesized in both neurons and activated glia. Pharmacological administration of estradiol and/or progesterone counteracts the exacerbation of astrocytes and microglia and constrains synthesis and release of pro-inflammatory cytokines (IL-1β, TNF-α…etc), whereas E2 and P4 association inhibits or reduce this response. Alternatively E2 may improve myelination enhancing proliferation/differentiation of oligodendrocytes which finally will favor axonal remyelination in the neuronal repair process. While the combination with P4 may damper the proliferation and differentiation of oligodendrocyte precursors (OLIG-P) challenging the recovery. It is not yet clear whether co-administration of E2/P4 disrupts the neural repair and neuroprotection that is demonstrated with estradiol alone. A full color version of this figure is available at https://doi.org/10.1530/JOE-18-0129.

added presence of progesterone diminished some of these observed beneficial effects. Thus, we have proposed that progesterone may be partially blocking positive effects of estradiol in the brain (Perez-Alvarez *et al.* 2015).

Conclusions

Beyond their roles in reproduction, the sexual hormones estradiol and progesterone play a pivotal role in brain functions including development, differentiation and neuroprotection. Estradiol and progesterone show similarities related to their receptors and mechanism of action; both have two main types of receptors that localize to different cellular compartments: nuclear receptors (with a delayed response) and cytoplasmic/membrane receptors (with a fast response). These two groups of receptors trigger different, complex mechanisms that are not fully understood. Estradiol and progesterone, when administered independently, have been described as neuroprotective agents in some neurodegenerative disease models, traumatic disease and even stroke. However, their combined application is controversial, as the effects are sometimes synergistic and other times antagonistic. The discrepancy between different research groups may be explained based on differences in onset of administration, ratio of hormone concentrations, duration of action and brain region studied. Furthermore, details about the interactions between estradiol and progesterone and the differential expression of their receptors in each brain region and are not well understood. According to current evidence, it is possible that they have a close relationship, co-regulating each other's receptors, levels in the brain and cross-interact during intracellular signaling. A deeper understanding of the mechanisms and relationship between both hormones is needed in order to clarify the discrepancies and allow for the design of effective therapies in the future.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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