

Estradiol and Progesterone Administration After pMCAO Stimulates the Neurological Recovery and Reduces the Detrimental Effect of Ischemia Mainly in Hippocampus

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Abstract Epidemiological studies have suggested a differential response, males versus female, in stroke incidence and prognosis. These divergences in brain response after damage are based mostly on hormonal differences. To date, estradiol and progesterone administered independently have demonstrated neuroprotection after ischemia in animal models. Nonetheless, contradictory results were revealed using a combined administration. In order to evaluate the effects of combinatorial treatment administered after ischemia induction, we used two different approaches: *in vivo* and *in vitro* models. Male rats which underwent permanent middle cerebral artery occlusion were treated with a combination of estradiol/progesterone at 6, 24 and 48 h after injury and sacrificed at 54 h post-ischemia. The rat brains were evaluated for reactive gliosis, NeuN-positive neurons, levels of synapse-associated proteins and activity levels of PI3K/Akt/GSK3/ β -catenin survival pathway. Also, primary cortical neurons were subjected to oxygen and glucose deprivation for 17 h and returned to a normal environment in the presence of estradiol or estradiol/progesterone. Cell viability was evaluated, and activity levels of the PI3K/Akt/GSK3/ β -catenin pathway. Our results

indicate that some beneficial effects of estradiol were abolished in the presence of progesterone, particularly in the cerebral cortex (core). However, the combinatorial treatment showed positive effects in the hippocampus.

Keywords Estradiol · Glia · Neuroprotection · OGD · Progesterone · Stroke

Introduction

Stroke, a complex neurodegenerative disease, is the leading cause of disability and the second cause of death in humans worldwide [1]. Brain injury following an ischemic insult involves a complex succession of events that cause varying degrees of cell damage depending, not only on the characteristics of the initial insult, but also on the duration of the occlusion [2]. It has been accepted that the cell death in the ischemic core, the most affected area, is triggered by necrosis, whilst in the peri-infarct region (penumbra) is caused mainly by apoptosis [3, 2, 4]. Until now, there is no pharmacological therapy for this disorder except tissue plasminogen activator, but its use is limited. The middle cerebral artery occlusion (MCAO) is the *in vivo* model most commonly used to mimic and analyze the ischemic stroke damage and also the potential treatments [5]. Epidemiological studies suggest that the stroke risk is lower and the prognosis of patients is better in premenopausal women than in men. Nonetheless, the incidence of cerebrovascular diseases increases in women after menopause, being more elevated than in men [1]. On the other hand, a gender-differential response after ischemic injury has been demonstrated in animal models of stroke [6, 7].

17 β -estradiol (E) and progesterone (P), the main female sex hormones, play a role in a multitude of physiological nervous processes acting both on neurons and in glial cells [8, 9]. They can cross the blood-brain barrier (BBB) and

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diffuse throughout the brain. However, they are considered as *neurosteroids* because they can be synthesized by the nervous system [10]. Nowadays, the neuroprotective effects of both hormones in different neurodegenerative diseases are well established [8, 9, 11]. Growing evidence strongly indicates that estradiol protects against ischemic stroke damage. In fact, estradiol administration before, during or after ischemia induction reduced the damage area in rodent models [12, 9]. Several mechanisms have been proposed to be involved in the estradiol neuroprotection against ischemia-induced damage, such as a reduction in pro-inflammatory events, glutamate-mediated excitotoxicity, oxidative damage and apoptosis [13–15]. However, only a few studies have examined the potential neuroprotective effect of progesterone, probably because the majority of the studies have focused on estradiol as the main source of neuroprotection seen in females. The neuroprotective effect of progesterone against ischemic brain damage includes decreasing infarct area, inflammation, BBB permeability and edema in animal models [16].

The detailed molecular mechanisms involved in neuroprotection by estradiol and progesterone are not completely understood. It has been described that both hormones exert their effects not only at the genomic level through the classical nuclear receptors, ‘slow responses’, but also by modulating cytosolic signalling cascades by interacting with membrane-localized and/or cytoplasmic receptors, ‘rapid response’ [11, 8]. These ‘cytoplasmic’ responses may play an important role in neural cells, especially in neuroprotection.

Moreover, the role of estradiol and progesterone has been described in synaptic plasticity [17], an important process in the recovery of brain function after damage [18, 19]. It has been reported that estradiol alters, through membrane receptors, the phosphorylation levels of synapsin I [20], a presynaptic protein that has a central role in neurotransmitter release, synaptic formation and maturation [19]. In addition, recent reports suggest an increase in postsynaptic density protein 95 (PSD95) expression, a postsynaptic protein located mainly at the glutamatergic synapses, mediated by estradiol [21].

To date, limited studies using a combination of estradiol and progesterone have been reported and the results obtained have been contradictory. Some point to the antagonistic effects between both hormones [22, 23, 17] whilst others report enhanced effects [24, 25].

In this study, we have analyzed the effect of post-ischemia estradiol and progesterone (E/P) treatment at physiological doses 54 h after injury. We performed two different experimental approaches using both *in vivo* (permanent middle cerebral artery occlusion, pMCAO) and *in vitro* (oxygen-glucose deprivation (OGD) in cortical neuronal primary culture) models. We have analyzed the effect of E/P post-ischemic treatments on *reactive gliosis* and NeuN-positive neurons. We have also evaluated the impact of this combined hormone therapy on the PI3K/Akt/GSK3/ β -catenin survival

pathway and on specific proteins implicated in synaptic plasticity and functionality (synapsin I, PSD95 and α -N-catenin). Our findings indicate that the addition of progesterone could abolish some of the beneficial effects observed previously by estradiol administration after pMCAO.

Materials and Methods

Animals

Adult male Wistar rats (8–12 weeks old, 250–310 g) obtained from the *vivarium* at Centro de Biología Molecular “Severo Ochoa” (CBM-SO) were maintained under 12-h light/12-h dark cycle and with access to food and water *ad libitum*. All the animal care protocols were conformed to the appropriate national legislations (RD 53/2013) and the guidelines of the European Commission for the accommodation and care of laboratory animals (revised in Appendix A of the Council of Europe Convention ETS123). All procedures have the approval of the Bio-Ethical committee of CBM-SO.

Surgery and pMCAO Induction

pMCAO was performed by right middle cerebral artery occlusion using an intraluminal suture as we previously described [13]. Anaesthesia was induced by intraperitoneal injection (ip) of a mixture of medetomidine (250 μ g/kg) and ketamine (75 mg/kg), accompanied by a subcutaneous injection of atropine (100 μ g/kg).

Hormonal Administration

A total of 32 male rats were randomly distributed into the following experimental groups: sham-vehicle (SV, $n=8$), ischemia-vehicle (IV, $n=8$), sham-estradiol/progesterone (SEP, $n=8$) and ischemia estradiol/progesterone (IEP, $n=8$). The animals received three doses of 0.04 mg/kg β -estradiol (Sigma, St. Louis, MO, USA, ref E8875) as we previously described [13], combined with 4 mg/kg progesterone (Sigma, St. Louis, MO, USA, ref P7556) [26, 27] in a total volume of 500 μ l. Intraperitoneal injections were administered at 6, 24 and 48 h after pMCAO induction. Groups IV and SV received 500 μ l of vehicle (1 % ethanol in saline). All animals were sacrificed 6 h after the last treatment.

Neurological Deficit Score

The neurological deficit score of each animal was measured before surgery, and at 6, 24, 48, 54 h after pMCAO induction, using the method described by Yrjänheikki [28]. A 7-point scale of neuromotor function was ranked as follows: grade 0, no spontaneous motion; grade 1, spontaneous circling towards

the paretic side; grade 2, circling towards the paretic side when pulled by the tail; grade 3, circling towards the paretic side when pulled and lifted by the tail; grade 4, consistently reduced resistance to a lateral push towards the paretic side; grade 5, consistent flexion of the forelimb contralateral to the injured hemisphere; and grade 6, normal extension of both forelimbs towards the floor when lifted. Other neurological anomalies were also recorded, such as alterations in balance, sensorial perception (acoustic, visual localization) and reflex responses (corneal, palpebral, postural correction). In this study, we have excluded all the ischemic rats that did not reach an ischemic damage index (IDI) of 2–3 at 6 h post-MCAO.

Western Blotting

Tissue samples were homogenized and analyzed by Western blots as we previously described [13]. Membranes were incubated overnight at 4 °C with the following primary antibodies: Akt (1:1000; Cell Signaling no. 9272), phospho-Akt^{Ser473} (1:500; Cell Signaling no. 9271), phospho-Akt^{Thr308} (1:1000; clone C31E5E, Cell Signaling no. 2965), β -catenin (1:800; BD Transduction Laboratories no. 610,153), β -tubulin (1:1000; clone TUB 2.1, Sigma no. T4026), β -actin (1:1000; clone AC-15, Sigma no. A5441), GSK3 (1:1000; Biosource no. 44–610), phospho-GSK3^{Ser21/9} (1:1000; Cell Signaling no. 9331), synapsin (1:500; Cell Signaling no. 2312), phospho-synapsin^{Ser9} (1:500; Cell Signaling no. 2311), PSD95 (1:1000; Cell Signaling no. 3450) and α -N-catenin (1:1000; clone C12G4; Cell Signaling no. 2163). Membranes were rinsed with PBS-Tween and incubated with appropriate secondary antibodies: anti-rabbit or anti-mouse IgG-HRP (Santa Cruz Biotechnology). The density of the immunoreactive bands was analyzed using Quantity One software (Bio-Rad; U-Max PowerLook 2100XL scanner) and the values were normalized to β -tubulin or β -actin levels detected in the same membrane and compared with control values (SV). The graphs represent mean \pm standard error of mean (\pm SEM) of 3–5 independent experiments.

Tissue Collection

For immunoblotting analysis, animals were sacrificed by exposure to CO₂ at 54 h after onset of pMCAO. The right cerebral cortex and hippocampus was dissected out and frozen at –80 °C for subsequent analysis.

For immunohistochemistry, the deeply anaesthetised animals using a mixture of medetomidine (375 μ g/kg) and ketamine (112.5 mg/kg) ip, were transcardially perfused with ice-cold 0.1 M phosphate-buffered saline (PBS) followed by 4 % paraformaldehyde in 0.1 M PBS, pH 7.4. After perfusion, the brains were removed and post-fixed overnight at 4 °C followed by cryoprotection using 30 % sucrose in PBS at 4 °C for

48–72 h. Finally, cryoprotected brains were embedded in Tissue-Tek medium (Sakura, Zoeterwoude, Netherlands). Coronal cryostat sections (25- μ m thick) were collected onto Superfrost Ultra Plus slides (Thermo Scientific, Braunschweig, Germany), air dried and stored at –20 °C. Sections located from +2 to –4 relative to bregma [29] were selected for immunohistochemistry/immunofluorescence.

Immunohistochemistry and Immunofluorescence

Immunohistochemical and immunofluorescence analyses were performed in a humidified chamber. The sections were permeabilized in PBS –0.25 % Triton X-100. After the tissue was incubated in blocking solution containing PBS –0.1 % Triton X-100 with 1 % horse serum. The slices were incubated overnight at 4 °C with the appropriate primary antibody in blocking solution: monoclonal mouse anti-gial fibrillar acidic protein (GFAP) (1:1000; Clon 4A11; BD Pharmingen no. 55632); rabbit anti-Iba1 (1:500; Wako no. 019–19741); monoclonal mouse anti-NeuN (1:100; Clon A60; Millipore MAB377). The sections were then washed with PBS –0.1 % Triton X-100 and incubated in PBS –1 % hydrogen peroxide. The slices were subsequently incubated at room temperature with the appropriate secondary antibody: biotinylated anti-mouse IgG or anti-rabbit IgG (1:200 in PBS –1 % horse serum or goat serum, respectively). After, the sections were incubated for 30 min with avidin-biotin-peroxidase complex from the Vector ABC Elite kit (VECTASTAIN, Vector laboratories Inc., Burlingame, CA, USA), followed by 15-min incubation with FAST 3 3 diaminobenzidine tablets (Sigma, D4168). After dehydration, the sections were cleared with xylol and mounted using Entellan New mounting medium (Electron Microscopy Sciences, Hatfield, PA, USA).

For immunofluorescence experiments, following incubation with the primary antibody, the sections were incubated with anti-mouse Alexa-555 (1:500; Invitrogen). The slices were mounted using Fluoromount-G (Southern Biotech). The immunostained slides were observed under an Olympus BX61 microscope and images captured using an Olympus DP50 camera.

Images Quantification

The intensity of immunostaining was quantified using Fiji image processing software [30]. For DAB-processed images (stained with GFAP and Iba-1), $\times 4$ -magnification images was used. After conversion of 16-bit, images was normalized subtracting background (*rolling=500 light*). The threshold was automatically selected (*AutoThreshold*) using the Moments method. To determine the size of the stained selected area, we analyzed particles (selecting size=100–5000 and circularity=0.10–1.00). The values were normalized to the total area of the tissue including in the picture that was

measured in each image. For NeuN quantification, $\times 10$ -magnification images were used. A grey-level threshold representing the boundary between background and positive staining was automatically selected by the software and then applied to measure the area of positive staining in each image. Prior to quantification, images were corrected using a variance filter (radius=1 pixel). To determine whether the immunostaining area changed in any experimental group, we quantified the positive staining area in NeuN images and these values were normalized to the total area of the photographed sections.

Cortical Primary Neurons Culture and Oxygen-Glucose Deprivation

Primary mouse neurons were prepared from 16-day-old Swiss mice embryos. A pool of cerebral cortices were dissected and enzymatically digested with 0.05 % trypsin-EDTA (Invitrogen) and 10 mg/ml DNase (Roche Applied Science) for 15 min at 37 °C, followed by trituration with fire-polished glass pipettes. The cells were plated on 24-well plates for viability and 6-well plates for Western blot analysis at a density of 2.5×10^5 cells/ml. The plates were previously pre-coated with 0.5 mg/ml poly-L-lysine (Sigma-Aldrich) in borate buffer. Neurons were cultured with neurobasal medium (NB) supplemented with 2 % B27 and 2 mM glutamine (Invitrogen), and maintained at 37 °C in a humidified 95 % air/5 % CO₂ atmosphere incubator. Five-day-old cultures were used for all experiments. The cultures contain almost 90–95 % of neurons and were negative to GFAP by western blot.

For oxygen-glucose deprivation (OGD), culture neurons were incubated in glucose-free Dulbecco's modified Eagle's medium (DMEM) and in a near-anaerobic incubator containing an atmosphere of 0.2 % O₂, 5 % CO₂ and 94.8 % N₂ at 37 °C. As control (non-OGD condition), DMEM media and normal incubator were used. After 17-h OGD, the cultures were returned to a normal environment; glucose-free media was replaced with NB/B27 media and cultures were returned to a 95 % air/5 % CO₂ incubator. Experimental parameters were assayed at 24 h following re-oxygenation/regeneration. Neuronal cultures were treated after OGD with β -estradiol (200 nM) and progesterone (20 μ M) during 24 h.

MTT Cell Viability Assay

Cell viability was examined by MTT assay that depends on the reduction of the tetrazolium salt MTT to formazan by living cells. Mouse primary neurons were cultured in 24-well plates. After treatment, a MTT solution (0.3 mg/ml) made in phenol red-free NB was added to the cells. After 1-h incubation at 37 °C, the MTT solution was aspirated and DMSO was added to the cells. Aliquots were transferred to a 96-well plate and absorbance measured at 540 nm in a plate

reader spectrophotometer (Opsys MR, Dynex Technologies). Results were expressed as percentages of the values obtained from the controls (non-OGD condition).

Statistical Analyses

GraphPad Prism software was used for all statistical analyses and all the data are presented as mean \pm standard error of mean (SEM). Statistical significance was estimated using a two-tailed Student's *t* test to compare differences between groups (SV vs IV; IV vs IEP). The neurological score was analyzed by two-way analysis of variance (ANOVA) with three groups (sham, IV and IEP), followed by Tukey's post hoc test for group comparisons between groups (SV vs IV and IV vs IEP). A value of $p \leq 0.05$ was considered statistically significant.

Results

Post-ischemia Administration of Estradiol/Progesterone Accelerates the Recovery of Neurological Status in Male Rats After pMCAO Induction

To investigate the effect of a combined hormone therapy administered after ischemic stroke, we treated pMCAO-induced rats at 6, 24 and 48 h with a mixture of estradiol/progesterone and monitored the behavioural scores. As Fig. 1 shows, all the animals attained the maximal ischemic damage index (IDI) before surgery was carried out. At 6 h post-surgery, immediately before the first treatment, the ischemic rats (IV and IEP groups) showed a neurological score significantly lower than the sham-operated animals (2.8 ± 0.374 vs 5.2 ± 0.374 ; $***p = 0.0002$). This showed that pMCAO

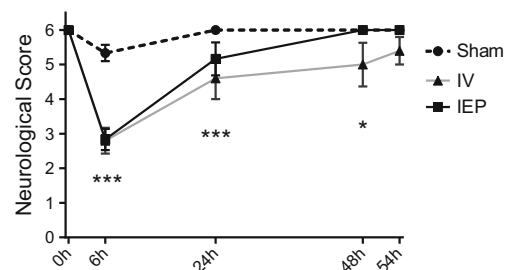


Fig. 1 Post-ischemic estradiol/progesterone combined treatment accelerates the recovery of maximum ischemic damage index (IDI). The IDI was measured using a 7-point scale neurological test. Zero corresponds to the lowest and 6 to the highest degree of neurological recovery. *Graphs* represent the neurological score of all animals analyzed at 6, 24, 48 and 54 h after ischemic onset. Data represent the mean \pm standard error of mean (SEM), the data were statistically significant at 6 h $***p = 0.0005$ (SV vs IV and SV vs IEP), at 24 h $***p = 0.0005$ (SV vs IV), At 48 h $*p = 0.03$ (SV vs IV) according to two-way ANOVA followed by Tukey's test. SV, Sham-vehicle; IV, pMCAO-vehicle; IEP, pMCAO-estradiol/progesterone

induction had a significant and consistent effect on the neurological status of the rats. At 24 h, the ischemic rats treated with the vehicle (IV) maintained significant lower values on IDI (4.6 ± 0.6 ; $***p=0.0002$). However, a unique injection of E/P induced a non-significant improvement on the neurological scores (5.1 ± 0.47). At 48-h post-pMCAO induction, the IV group conserved a reduced IDI of 5 ± 0.63 ($*p=0.031$) whilst the IEP group recovered the maximum score index. Finally, all the experimental groups achieved the maximum IDI before sacrifice.

The Estradiol/Progesterone Treatment After pMCAO Reduces the Reactive Gliosis Induced by Ischemia

After MCAO induction reactive gliosis has been reported [31, 13]. It is characterized by the activation and proliferation of microglia and astrocytes especially in regions near to the ischemic core and exerts both beneficial and detrimental effects on brain damage after injury. To further assess the effect of E/P treatment on reactive gliosis after pMCAO, we examined this phenomenon in coronal sections immunostained with astrocyte-specific and microglia-specific antibodies, GFAP and Iba-1, respectively (Fig. 2). We focused on the parietal and piriform cortex where we observed a larger proliferation of these cells after pMCAO induction.

As we have already described [13], the GFAP-positive marked area in IV group was significantly higher than in the same area of SV group (4.81 ± 0.91 vs 1.67 ± 0.27 %; $p \leq 0.01$; Fig. 2a). Additionally, a similar pattern was found when we examined the Iba-1-positive stained area (10.62 ± 1.40 vs 5.99 ± 1.17 %; $p \leq 0.05$; Fig. 2b). As expected, these findings indicate a marked accumulation of astrocytes and microglia in particular, near to the damaged area 54 h after ischemia onset. The administration of three doses of E/P after pMCAO induced a decrease in the percentage of GFAP-positive and Iba-1-positive areas in the ischemic territory (Fig. 2a, b; right panels). The percentage of Iba-1 marked surface decreased significantly to basal levels (6.09 ± 0.5 %; $p \leq 0.05$), but the percentage of GFAP-marked surface decreased without reaching the levels of SV group (2.38 ± 0.8 %; $p \leq 0.05$). This result suggests that post-pMCAO E/P treatment could reduce the *reactive gliosis* triggered by ischemia with a slightly greater effect on microglia than in astrocytes.

Estradiol/Progesterone Treatment Increases the Amount of NeuN-Positive Cells in CA2-CA3 but Not in the Cortex

After cerebral blood flow deprivation, some neurons, especially those located in the ischemic core, initiate a detrimental process that could induce cell death. The next step was to

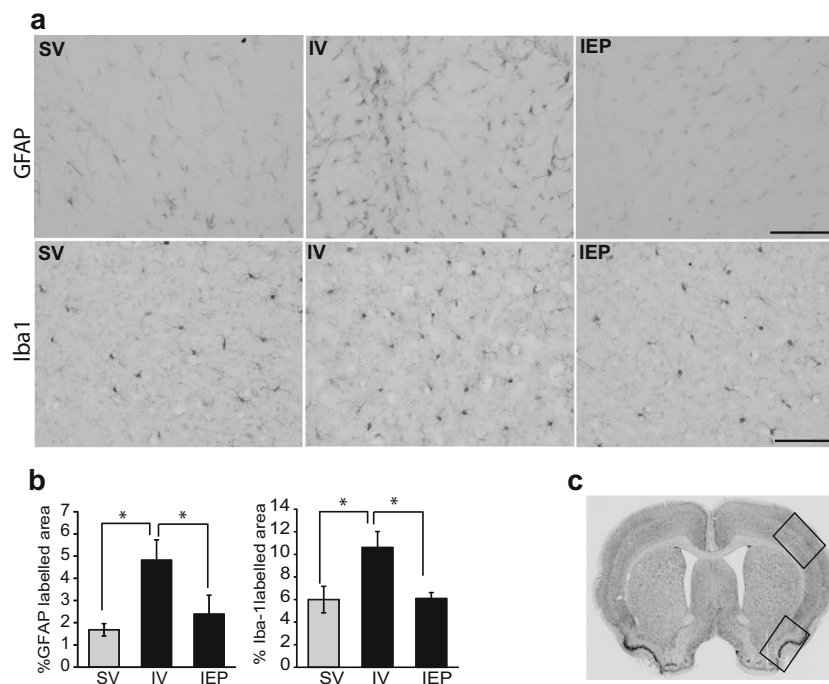


Fig. 2 The *reactive gliosis* is reduced after E/P treatment in the ischemic area. The *reactive gliosis* induced after 54-h pMCAO was measured in coronal brain sections by immunohistochemistry using GFAP and Iba-1, markers of astrocytes and microglia, respectively. (a) Representative coronal sections corresponding to the injured area (bregma 0.48) of sham-vehicle (SV), pMCAO-vehicle (IV) and pMCAO-estradiol/progesterone (IEP) rats are shown. GFAP (a upper panel) and Iba-1 (b bottom

panel). **b** The graphs represent the percentage of marked area expressed as the percentage of labelled tissue with respect to the total area of the photographed sections. Staining was quantified in at least six serial coronal sections of each animal (from bregma 2.04 to -0.48). **c** A Nissl-stained section showing the affected areas selected for GFAP/Iba-1 quantification, as indicated by rectangles. $*p \leq 0.05$; scale bar = 100 μ m

evaluate the impact of this combined hormone treatment on neuronal cells after pMCAO induction. It was revealed that the percentage of NeuN-positive marked area in IV group was significantly lower compared with the SV group (12.66 ± 0.24 vs 16.53 ± 1.2 %; $p \leq 0.05$, Fig. 3) when the somatosensory cortex (ischemic core) was analysed by immunofluorescence. In addition, post-pMCAO treatments with a mixture of E/P did not cause any effect on the percentage of NeuN-positive marked area, since we detected similar levels in the IV group (13.62 ± 0.73).

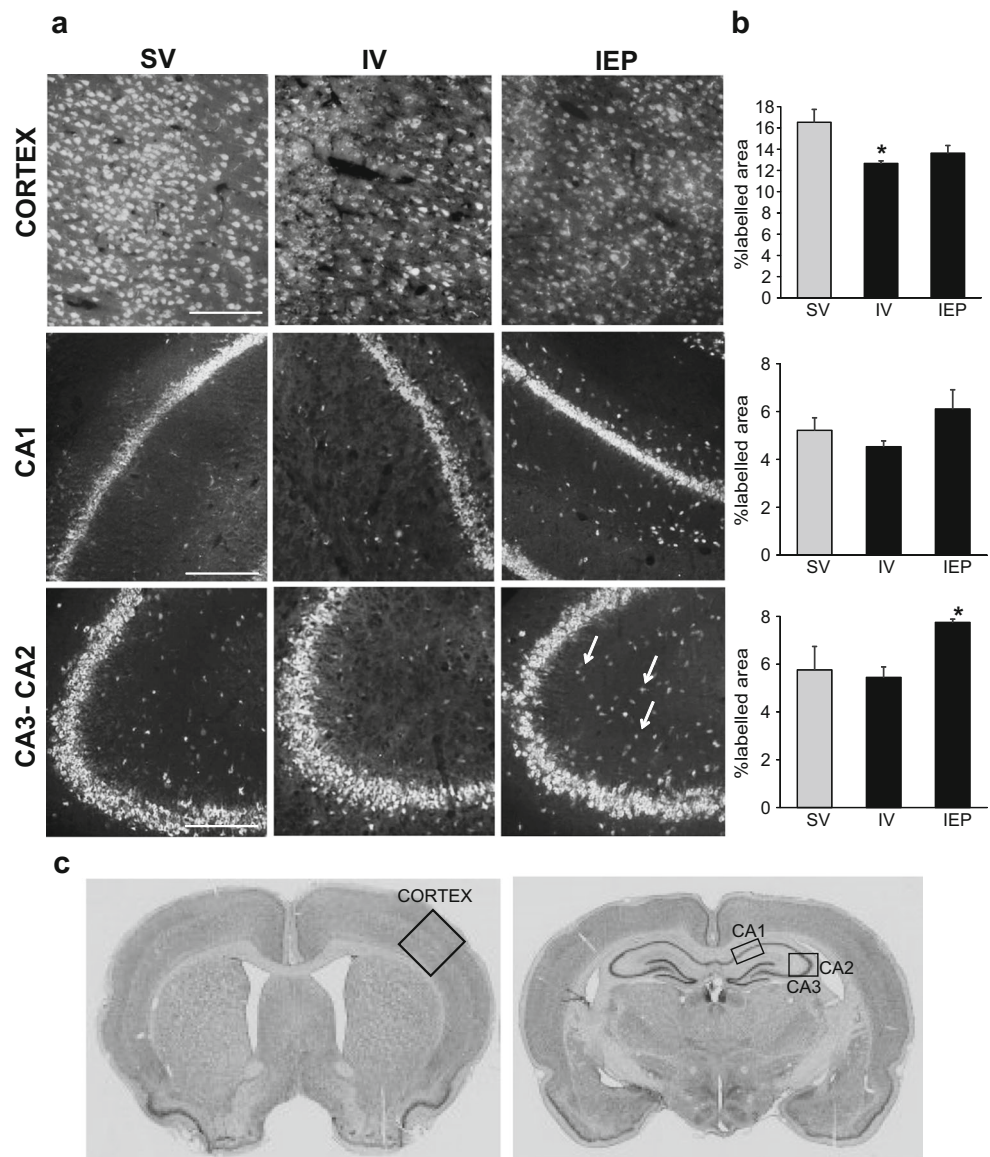
NeuN staining in the hippocampus CA1, and CA2-CA3 region, showed non-significant differences between SV ($CA1=408 \pm 47.3$; $CA2-CA3=451 \pm 54$) and IV groups ($CA1=360.7 \pm 16.8$; $CA2-CA3=314.6 \pm 82$; Fig. 3). These findings confirmed that the hippocampus did not suffer the same degree of injury as the somatosensory cortex 54 h after

pMCAO induction. Remarkably, E/P treatments induced a significant increase in the percentage of NeuN-staining area in internal region around CA2-CA3 (584.5 ± 9.9 ; $p \leq 0.01$) but this effect was not found in CA1 region (482.2 ± 56.9). This rise in NeuN staining was observed especially in *stratum lucidum* and *radiatum* of the hippocampus, just under the pyramidal cell layer (Fig. 3 arrows).

Effect of Estradiol/Progesterone on Synapse-associated Proteins

To evaluate more specifically this combined treatment on neurons, we analyzed some proteins directly associated with synaptic structure and function, highly sensitive to ischemia [32]. We focused on a member of the catenin family (α -N-

Fig. 3 The amount of NeuN-positive cells in CA2-CA3 increases after post-ischemic treatment with estradiol/progesterone. **a** Representative coronal sections corresponding to ischemic core (somatosensory cortex; bregma 0.12), and hippocampus (CA1 and CA2-CA3; bregma -3.36) of sham-vehicle (SV), pMCAO-vehicle (IV) and pMCAO-estradiol/progesterone (IEP) rats. **b** The bar graph represents the percentage of marked area normalized to the total area of the photographed sections. NeuN staining was quantified in at least six serial coronal sections of each animal, from bregma 2.04 to -0.48 (cortex) and bregma -2.76 to -3.84 (hippocampus). Arrows show NeuN-positive neurons in stratum lucidum/radiatum. **c** Nissl-stained sections showing the selected areas for NeuN quantification, as indicated by rectangles. Data represent mean \pm standard error of mean (SEM). * $p \leq 0.05$; scale bar = 100 μ m



catenin) [32], a member of postsynaptic density protein (PSD) family, PSD95; and a presynaptic protein (synapsin).

To demonstrate further the impact of E/P treatment on neurons, the levels of these proteins were checked 54 h after pMCAO induction (Fig. 4). The total levels of α -N-catenin did not show any significant differences between the experimental groups neither in the cortex nor the hippocampus (Fig. 4a, e). The PSD95 levels exhibited a slight increase in IV group in the cerebral cortex but not in the hippocampus (Fig. 4b, f). The administration of both hormones caused a non-significant reduction of the pMCAO-increased level (Fig. 4b, f). The total levels of synapsin were not altered after ischemia induction (Fig. 4c, g) in both regions studied. However, after 54-h pMCAO, the p-synapsin^{Ser9} levels were significantly increased in both the cortex and the hippocampus when compared with the SV group, 130.12 ± 19 and 117.8 ± 4.6 %, respectively. The E/P treatment induced a reduction in these pMCAO-increased levels, but this was only statistically significant in the hippocampus (Fig. 4d, h).

Estradiol/Progesterone Treatment Induces a Recovery in PI3K/Akt/GSK3/ β -catenin Survival Pathway in the Hippocampus but not in the Cortex

Although the detailed molecular mechanisms by which both estradiol and progesterone enhance neuronal survival are not clearly understood, it has been established that both hormones activate multiple intracellular signalling cascades. The PI3K/Akt/GSK3/ β -catenin survival pathway is one of the well-described mechanisms, implicated in the neuroprotection triggered by estradiol and progesterone [33, 34]. Recently, we reported that estradiol treatments reverted the pMCAO-induced downregulation of the PI3K/Akt/GSK3/ β -catenin pathway in the parietal cortex, reflecting an important neuroprotective effect in this ischemic area [13]. Therefore, in this study, we investigate whether the combination of estradiol and progesterone could modify the neuroprotective effect identified with estradiol.

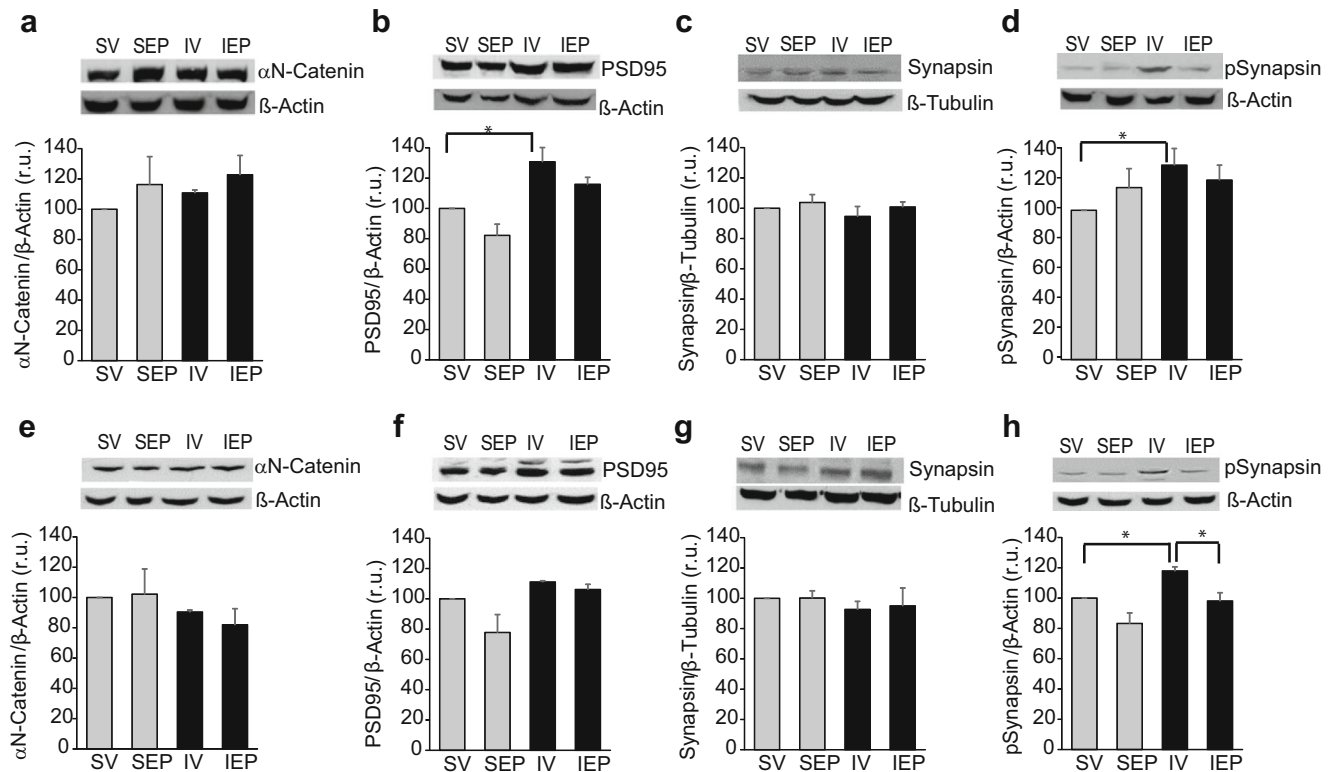


Fig. 4 Effect of post-pMCAO-estradiol/progesterone treatment on synapse-associated proteins. The total levels of α -N-catenin (a, e), PSD95 (b, f) and synapsin (c, g) as well as the phosphorylated levels of p-synapsin^{Ser9} were measured by Western blots of the ipsilateral cerebral cortex (a–d) and hippocampus (e–h) homogenates. The bar graphs represent the levels of these proteins normalized to β -actin/ β -tubulin. A representative blot is shown above each graph. The bars represent the mean of 3–5 independent experiments. The total levels of α -N-catenin both in the cortex and hippocampus (a, e, respectively) did not show significant variations between the different experimental groups. The pMCAO (IV group) induced a significant increase in PSD95 levels in

the cortex but not in the hippocampus (b and f, respectively). The estradiol/progesterone treatment did not significantly alter the pMCAO-induced levels. In both the cortex and hippocampus, the total synapsin levels (c, g, respectively) did not change in any experimental group. But, the p-synapsin^{Ser9} levels increased after pMCAO induction (IV group) in both the cortex (d) and hippocampus (h). Estradiol/progesterone treatment (IEP group) reduced p-synapsin^{Ser9} down to control levels only in the hippocampus (h). Data were expressed as the mean \pm SEM * $p < 0.05$; $n = 5$ animals per group. SV, Sham-vehicle; SEP, Sham-estradiol/progesterone; IV, pMCAO-vehicle; IEP, pMCAO-estradiol/progesterone; r.u., relative units

The effect of E/P treatment on Akt and GSK3 activities was examined by Western blotting using phospho-specific antibodies against the active/inactive forms of these kinases. The Akt activation was measured using pAkt^{Ser473} and pAkt^{Thr308} specific antibodies, the pGSK3^{Ser21/9} antibody can reflect the degree of inactivation of GSK3. Activate Akt can phosphorylate GSK3 in Ser21/9 residues reducing its kinase activity. We also checked the total amount of Akt and GSK3 using specific antibodies against these proteins. The effect of E/P treatment on β -catenin levels was analyzed using a specific antibody.

Firstly, we checked the activation status of this pathway in the parietal cortex. Unaltered levels of total Akt, GSK3 and β -catenin were found in all the experimental groups (Fig. 5) indicating no effect of the treatment on the total levels of these proteins. As we found in our previous study, the levels of pAkt^{Ser473}, pAkt^{Thr308} and pGSK^{Ser21/9} decreased significantly in cortical areas at 54-h pMCAO induction; indicating a

downregulation of this survival pathway (Fig. 5b, c and e). The degree of reduction in the pAkt epitopes was different, with pAkt^{Ser473} affected more ($40\pm 15\%$ of SV levels; $p\leq 0.01$) than pAkt^{Thr308} ($60\pm 5.7\%$ of SV levels; $p\leq 0.01$). The treatment using E/P did not significantly recover the pAkt^{Ser473} levels, but enhanced the levels of pAkt^{Thr308} which reached $85\pm 5.3\%$ of the SV levels ($p\leq 0.01$; Fig. 5c). The levels of pGSK3^{Ser21/9}, showed a reduction of $59\pm 9.7\%$ vs SV levels (Fig. 5e; $p\leq 0.05$). In this case, E/P administration was not able to recover the activity levels of this kinase.

On the other hand, analysis from the hippocampus samples obtained from pMCAO-induced animals exhibited a different response to the E/P treatments. As we observed in the parietal cortex, the total levels of Akt, GSK3 and β -catenin did not show any significant variation between the experimental groups (Fig. 6a, d and f, respectively). A significant reduction of Akt activity after pMCAO was inferred by the reduction of pAkt^{Ser473} ($37\pm 4.3\%$ reduction vs SV levels; $p\leq 0.01$), and

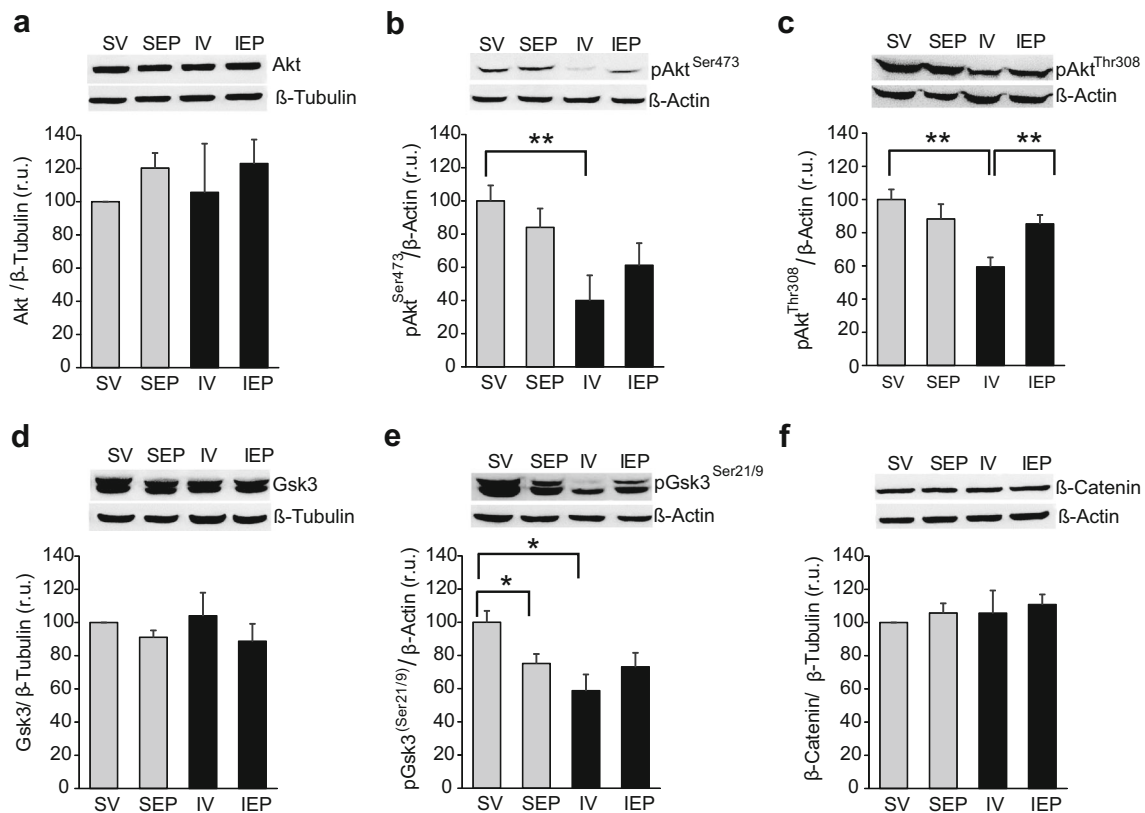


Fig. 5 The estradiol/progesterone treatment did not recover the downregulation of the PI3K/Akt/GSK3/ β -catenin pathway by ischemia in the parietal cortex. The activity levels of the PI3K/Akt/GSK3/ β -catenin pathway in the cortex was examined in homogenates from the ipsilateral cerebral cortex using phospho-specific antibodies against active/inactive forms of Akt/GSK3 kinases, respectively. The bar graphs represent the total levels of Akt (a), pAkt^{Ser473} (b), pAkt^{Thr308} (c), total GSK3 (d), pGSK3^{Ser21/9} (e) and β -catenin (f) normalized to β -actin/ β -tubulin. A representative blot is shown above each graph. The data represent the means of 3–4 independent experiments. No changes in the total Akt,

GSK3 and β -catenin levels were observed in any of the experimental groups (a, d and f). The pMCAO (IV group) induced a significant reduction in pAkt^{Ser473} (b), pAkt^{Thr308} (c) and pGSK3^{Ser21/9} (e). The estradiol/progesterone treatment (IEP group) did not induce any significant changes compared with the vehicle-treated group (IV) in pAkt^{Ser473} (b) and pGSK3^{Ser21/9} (e). However, estradiol/progesterone treatment partially recovered the pAkt^{Thr308} (c) levels. The data are expressed as the mean \pm SEM; * $p\leq 0.05$ ** $p\leq 0.01$; $n=5$ rats per group. SV, Sham-vehicle; SEP, Sham-estradiol/progesterone; IV, pMCAO-vehicle; IEP, pMCAO-estradiol/progesterone; r.u., relative units

pAkt^{Thr308} (66 ± 10 % reduction vs SV levels; $p \leq 0.05$) phospho-epitopes. In this case, the E/P treatments caused a significant increase in the levels of pAkt^{Ser473} and pAkt^{Thr308}, suggesting that E/P administration was able to revert the inactivation pMCAO-induced on Akt in the hippocampus. Similar findings were found when the GSK3 activity in this area was evaluated. IV rats showed an important decrease in the pGSK3^{Ser21/9} levels (38.5 ± 6.5 % reduction vs SV levels; $p \leq 0.01$); but E/P administration partially recovered GSK3 phosphorylation, reaching a value of 81.88 ± 5.5 % of SV levels ($p \leq 0.05$; Fig. 6e).

Progesterone Addition After Oxygen-glucose Deprivation Induction (OGD) Avoids the Estradiol Effects on Neuron Viability and PI3K/Akt/GSK3/ β -catenin Activity

To confirm that some of *in vivo* results are due to a neuronal response to the hormones, we subjected primary neuronal cultures to periods of OGD and we determined

cell viability at 24 h by MTT assay. Preliminary experiments using cells deprived of oxygen and glucose for various intervals (1 to 17 h) were carried out. The cells were analysed 24 h after incubation with the regeneration medium and the results indicated that a period of 17-h OGD caused a substantial, 50 % reduction in neuronal viability (data not shown). We analysed the effect of the regeneration medium supplemented with estradiol and estradiol/progesterone treatments after OGD (Fig. 7a). The results showed that the presence of estradiol at 200 nM induced a significant increase on neuronal viability, but the addition of progesterone at 20 μ M impeded this estradiol-induced effect.

Secondly, we examined the effect of OGD and ovarian hormone treatments on the PI3K/Akt/GSK3/ β -catenin pathway testing Akt and GSK3 activity using a Western blot (Fig. 7b). An analysis of the cell extracts from cortical neurons exposed to OGD revealed a downregulation of this pathway, reflected by a significant decrease in pAkt^{Ser473}, pAkt^{Thr308}

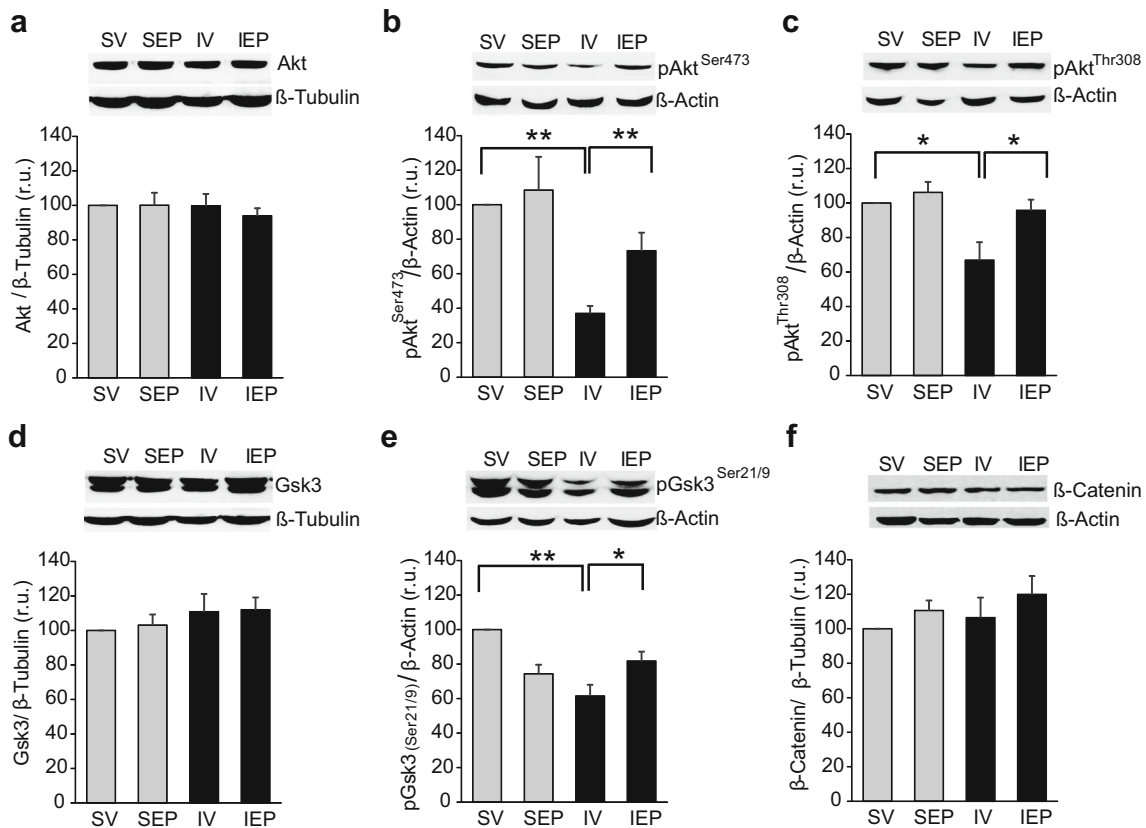


Fig. 6 The activity levels of Akt and GSK3 were recovered by estradiol/progesterone treatment post-pMCAO in the hippocampus. Akt and GSK3 activity levels were measured in the ipsilateral hippocampus by Western blotting using phospho-specific antibodies against active/inactive forms of both kinases. The bar graph represent the levels of total Akt (a), pAkt^{Ser473} (b), pAkt^{Thr308} (c), total GSK3 (d), pGSK3^{Ser21/9} (e) and β -catenin (f) normalized to β -actin/ β -tubulin. A representative blot is shown above each graph. The data represent the mean of 3–4 independent experiments. The levels of pAkt^{Ser473} (b), pAkt^{Thr308} (c) and

pGSK3^{Ser21/9} (e) showed a significant decrease compared with the SV group after ischemia induction. The treatment using estradiol/progesterone (IEP group) significantly recovered the levels of pAkt^{Ser473} (b), pAkt^{Thr308} (c) and pGSK3^{Ser21/9} (e). Total levels of Akt (a), GSK3 (d) and β -catenin (f) did not change in any experimental group. The data were expressed as the mean \pm SEM; * $p \leq 0.05$ ** $p \leq 0.01$; $n = 5$ rats per group. SV, Sham-vehicle; SEP, Sham-estradiol/progesterone; IV, pMCAO-vehicle; IEP, pMCAO-estradiol/progesterone; r.u., relative units

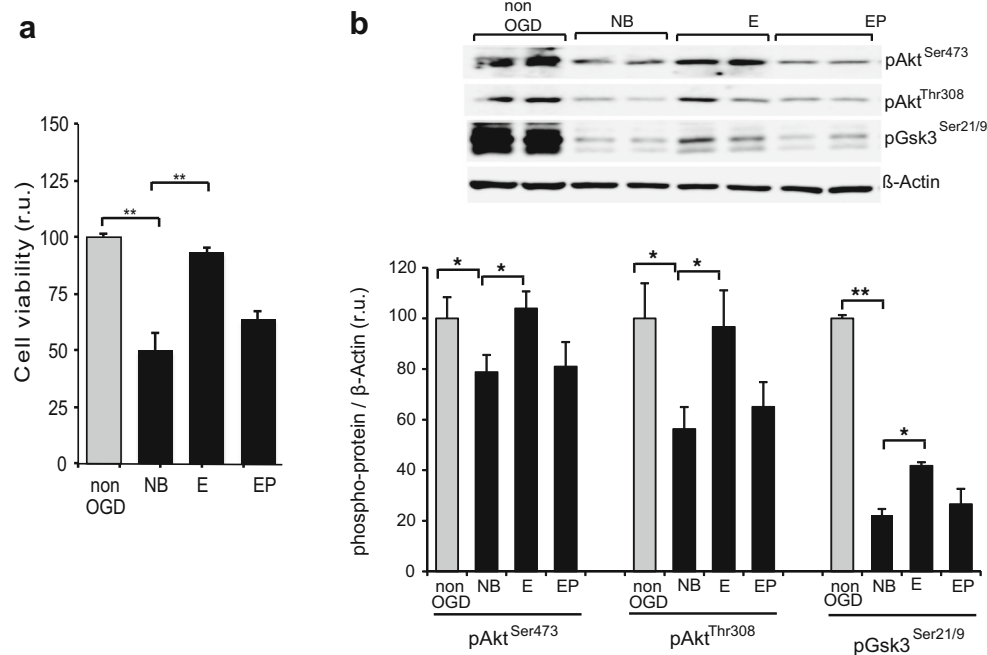


Fig. 7 Progesterone prevents the beneficial effect of estradiol in the cortical neurons after OGD. After overnight OGD, estradiol, estradiol/progesterone or vehicle were applied for 24 h. **a** MTT assay to assess cell viability after 17-h OGD and 24-h regeneration in the presence of estradiol, estradiol/progesterone or vehicle (NB). The addition of progesterone attenuated the estradiol-induced increase in cell viability. Data are expressed as the percentage values for non-OGD condition ($n=6$). **b** pAkt^{Ser473}, pAkt^{Thr308} and pGSK3^{Ser21/9} levels after 17-h OGD following regeneration for 24-h. Estradiol treatment significantly increased the

OGD-induced reduction in pAkt^{Ser473}, pAkt^{Thr308} and pGSK3^{Ser21/9} levels. The presence of progesterone abolished estradiol-induced effect. The bar graphs represent the quantification of phospho-proteins levels normalized to β -actin levels and are expressed as a percentage of the values of the controls (non-OGD). Bars represent the means \pm SEM of three samples per group. A representative blot is shown above the bar graph. (* $p < 0.05$, ** $p < 0.01$). NB: regeneration in presence of vehicle; E: regeneration in presence of estradiol; E/P: regeneration in the presence of estradiol/progesterone; r.u., relative units

and pGSK3^{Ser21/9} levels (78.7 ± 11 , 56.3 ± 15 and 22.2 ± 4.3 % compared with the non-OGD controls, respectively).

The addition of estradiol to the regeneration medium for 24 h abolished the Akt inactivation caused by the OGD but did not completely abolish the decrease in pGSK3^{Ser21/9} levels. Moreover, the combination of E/P resulted in a non-significant variation in the pAkt^{Ser473}, pAkt^{Thr308} and pGSK3^{Ser21/9} levels compared with the OGD condition.

Discussion

At the current time, there are contradictory data in the literature regarding the neuroprotective effects of combined hormone administration compared with the treatment of estradiol or progesterone alone. It has been described that progesterone reduces the oestrogen-mediated neuroprotection against excitotoxicity [35] and inhibits some beneficial effects of estradiol in animal models [22]. However, epidemiological data obtained from humans and evidence from animal models of experimental stroke, suggest a neuroprotective effect of endogenous ovarian hormones against the cerebral ischemic damage [1, 6]. With regard to stroke models, there are only a

few articles that use the combined treatment and point to the beneficial effects preventing cell damage, improving behavioural recovery and reducing the oxidative stress [25, 24, 36]. The majority of these studies have been performed using transient MCAO (tMCAO), where focal blood flow deprivation for a short time, from 30 min to 2 h, is followed by reperfusion. Some researchers believe that permanent MCAO (pMCAO), where reperfusion is not permitted, has a greater clinical relevance than tMCAO [3]. In addition, most studies have established the potential of sexual hormones as preventive agents, treating animals before ischemic insult [37, 25, 36]. However, exploring the effects of these hormones administered after ischemic injury is more relevant and poorly investigated.

Our present data showed that the addition of progesterone combined with estradiol avoided some of the protective effect of estradiol both in vivo (pMCAO) and in vitro (OGD). In fact, we previously described that the administration of three injections of estradiol (0.04 mg/kg) after pMCAO, attenuated the ischemic injury in neuronal and glial cells, especially in the parietal cortex [13]. We evaluated whether progesterone combined with estradiol treatments modified the neuroprotective effects observed using estradiol alone. Six hours after pMCAO induction, a significant decrease in the IDI score

was found. Administration of E/P showed a trend towards accelerating the recovery of maximum IDI. The maximum neurological score in the IEP group was recovered earlier than in the IV group, suggesting an improvement in sensory-motor functions after the E/P treatment. These results are consistent with those obtained by others 24 h after ischemia onset in a tMCAO model [25, 38].

It has been well-established that astrocytes and microglia activation after ischemia is an adaptive response to regulate the neuronal death because they play a key role in repairs in the CNS [34]. There is evidence that astrocytes express all types of receptors for both steroid hormones [39, 40]. However, the expression of these receptors in microglia has not been well characterized. Several studies have indicated the presence of estradiol receptors in resting and activated microglia [41] but until now, the presence of classical progesterone receptors have not been detected in any physiological state [34]. In this study, we found that after 54-h pMCAO induction, the amount of astrocytes and microglia increased significantly compared with the SV group. The administration of three doses of E/P starting at 6 h post-ischemia decreased reactive gliosis, measured by the expression of GFAP and Iba-1. These data concur with our recently published work, using estradiol alone [13]. An identical reduction in Iba-1 levels was found with E/P treatment, indicating that it could mainly be mediated by the direct action of estradiol, because classical progesterone receptors have not been identified in microglia. However, the reduction in GFAP levels after E/P therapy was slightly lower than with estradiol alone [13]. This seems to indicate that progesterone could be partially blocking some of the effects of estradiol in astrocytes.

After blood flow deprivation, the neurons localized in the area affected suffered detrimental processes which triggered cell death. In the MCAO model, the striatum and the parietal cortex are considered to be the ischemic core and the hippocampus a region that suffers secondary damage [5]. In order to evaluate the degree of neuronal damage after pMCAO induction and the effect of E/P administration, we stained coronal sections with a neuron-specific antibody, NeuN. Our results revealed that the percentage of NeuN-positive labelled area in the parietal cortex was significantly decreased after 54-h pMCAO induction, but no significant variations were found in the hippocampus (CA1 and CA2-CA3) compared with the SV group. E/P treatments after an ischemic insult had no impact on neuronal numbers in the parietal cortex. The effects observed are somewhat similar to those obtained using an in vitro OGD model with primary cortical neurons. Neuronal cultures subjected to OGD conditions for 17 h decreased the cell viability by 50 %. The treatment using E/P did not cause any significant variation over the OGD group. Whilst the presence of estradiol in the regeneration media improved the cell viability to control levels (non-OGD). This suggests that, in our paradigm, progesterone could be blocking some of the

neuroprotective effects of estradiol. These findings were consistent with previous results obtained by Jayarama and Pike using cortical primary neurons [23]. They demonstrated the inhibitory effects of progesterone on estradiol-mediated neuroprotective actions and suggested that this effect could be mediated through downregulation of ER α and ER β expression. However, it has been described that E/P administration after reperfusion induces a reduction of approximately 70 % of the infarction volume (measured by hematoxylin-eosin staining or by NeuN immunohistochemistry) at 2 weeks after insult, using a tMCAO model [38]. This apparent discrepancy with our findings could be due to some factors that may strongly affect the degeneration/regeneration of injured brain areas: duration of artery occlusion; reperfusion vs non-reperfusion; hormonal doses and ratio used (E/P=0.025/10 vs 0.04/4 mg/kg); the time at which the first dose is administered (after reperfusion vs 6 h after the onset of ischemic insult); and the time when the tissue was analyzed (2 weeks vs 54 h after MCAO induction). It is well known that reperfusion, depending of damage period, help tissue regeneration. In our model, reperfusion is not allowed and therefore, the parietal cortex is more affected that in a tMCAO model. Also, the timing of pharmacological intervention affect neuronal rescue, an early intervention help to improve neuronal recovery. All these factors did not permit a straight comparison between our model system and this reported by Ulbrich et al. [38].

Nonetheless, treatments using E/P after pMCAO induced a significant increase in the percentage of NeuN-positive labelled area in CA2-CA3, with respect to the control, mostly in the *stratum lucidum* and *radiatum*. We also found that the NeuN-positive neurons in these layers decreased after pMCAO, whereas the quantity of neurons in the CA2-CA3 pyramidal layer (IV group) was similar to the SV group. The presence of some interneurons in the *stratum lucidum* implicated in the local circuits modulation has been described [42]. These neurons could be particularly vulnerable to ischemia and their degeneration affects the CA3 pyramidal neurons. Consequently, it could cause a delayed degeneration of the CA1 pyramidal cells [42]. We suggest that the increment in NeuN-positive neurons on *stratum lucidum* induced by E/P treatment could be due to an increase in NeuN expression in pre-existing interneurons. Additional experiments will be required to further confirm this hypothesis and finally decipher the E/P effect in the hippocampi.

It has been reported that the PI3K/Akt/GSK3/ β -catenin survival pathway is downregulated after an ischemic insult [33, 13]. In this and in our previous work, we have shown that ischemia induced a diminution of the Akt activity (determined by levels of phosphorylated residues: Ser-473 and Thr-308) which produces a reduction in pGSK3^{Ser21/9} levels, associated with an activation of GSK3 activity. We previously reported that estradiol, administered after pMCAO, recovered the Akt

activity predominantly in the cerebral cortex and to a lesser extent in the hippocampus, which correlates with an increment of GSK3 inhibition [13].

These results are in agreement with those obtained using our *in vitro* OGD model. The addition of E after OGD induced a recovery in Akt activity with the subsequent increase in pGSK3^{Ser21/9} levels, which may mediate the recovery of neuronal viability described above. Our *in vivo* and *in vitro* results are fully in agreement and enable us to postulate an important modulatory role of the estradiol-dependent PI3K/Akt/GSK3 pathway after ischemia. Administration of three doses of E/P after pMCAO slightly recovered the pAkt^{Thr308} epitope but not pAkt^{Ser473} in the cerebral cortex. This correlates with the effect observed in the pGSK3^{Ser21/9} levels and therefore maintained the GSK3 activity as seen in the IV group. Moreover, *in vitro* experiments using cortical neurons indicated that E/P treatment did recover either Akt activity or the degree of viability in the cortical neurons. Thus, both our present and previous work support the hypothesis that the post-pMCAO administration of estradiol reduces some of the negative effects of ischemic injury in the parietal cortex, both *in vivo* and *in vitro*, acting as a recovery agent. However, the presence of progesterone abolished some of the beneficial effects observed.

In the hippocampus, E/P treatments induced a recovery in the Akt activity levels, as demonstrated by the significant increase of pAkt^{Ser473} and pAkt^{Thr308} with respect to IV. This correlates with a significant increase in the pGSK3^{Ser21/9} levels and therefore an inhibition of GSK3 activity which can improve cell survival. In a previous report, we described that post-pMCAO-estradiol treatments were unable to reduce the GSK3 activity in the hippocampus [13]. The phosphorylation of both Akt-residues is necessary for the maximal Akt activity [43]. Different kinases are implicated in the phosphorylation of each residue: PDK1 (Thr-408) and mTORC2 (Ser-473) although the regulatory mechanisms, and physiological importance of each phosphorylation site, are still not fully understood [43]. According to our data, we hypothesize that post-pMCAO E/P treatment modulated PDK1 in the cortex and hippocampus, but mTORC2 only in the hippocampus through a mechanism that has not been unidentified. The results obtained in NeuN-positive cells suggests that E/P induced upregulation of pAkt^{Thr308} which occurred in the cortex, probably mediated by PDK1, but was not able to reach the Akt/GSK3 activity levels necessary for the recovery of the neurons in the ischemic core. However the pAkt^{Thr308} and pAkt^{Ser473} upregulation in the hippocampus (reflecting full activation) was able to achieve the suitable activity levels of Akt/GSK3 preventing the pMCAO-induced neuronal loss.

Therefore, we suggest that the combined administration of E/P reduces some of the beneficial effects triggered by

estradiol, mediated in part by the survival pathway PI3K/Akt/GSK3/ β -catenin, in the cerebral cortex. By contrast, this survival pathway is upregulated by E/P in the hippocampus, as shown by the increased levels of pAkt^{Thr308}, pAkt^{Ser473} and pGSK3^{Ser21/9}. The apparent discrepancy in the E/P effects observed in the cortex and hippocampus may be explained by variations in the ratio between progesterone/estradiol receptors between both cerebral territories. In a non-pathological condition, cortical and hippocampal neurons exhibits differences in expression of estradiol and progesterone receptors [44, 45]. These differences could be exacerbated by an ischemic insult because both territories are affected differently by pMCAO and the underlying cause of damage is not the same. In fact, after MCAO, the parietal cortex suffers a dramatic reduction in the glucose and oxygen supply whereas hippocampus underwent damage mainly by deafferentation. Nevertheless, further studies are required to reveal the detailed mechanism of E/P interactions.

In summary, we have used two experimental approaches and examined the effects of combined treatment using E/P after pMCAO induction. We have found that E/P administration after pMCAO induction (1) accelerated the recovery of neurological score in male rats; (2) reduced the degree of *reactive gliosis*; (3) did not avoid the neuronal loss in cerebral cortex, but induced an evident response of the neurons in the *stratum lucidum/radiatum* of the hippocampus CA2-CA3 region; (4) caused a significant recovery in the PI3K/Akt/GSK3/ β -catenin pathway in the hippocampus but not in the cerebral cortex. However, this hormonal treatment had no remarkable impact on synapse-associated proteins at this time.

A complementary approach was performed *in vitro* exposing primary neurons to OGD. We found that estradiol treatments recovered neuronal viability and recuperated the PI3K/Akt/GSK3 pathway downregulated by OGD. Moreover, the combination of progesterone and estradiol avoided the protective effect of estradiol. In conclusion, we propose that the post-ischemia administration of estradiol can reduce some neurodegenerative processes associated with ischemic damage in the cortex, both *in vivo* (pMCAO) and *in vitro* (OGD). But the presence of progesterone abolished the beneficial effects in the cortex.

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Conflict of Interest The authors declare that they have no conflict of interest.

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