Estetrol and Its Effects on the Damaged Brain

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4.1 Development of the Nervous System

Development of the nervous system in humans during pregnancy is passing through critical and complex periods of morphological and functional differentiation. During ontogenesis newly developed structures of the nervous system, which differ by function and localization, are unified in one complete functional system.

Main steps in human organogenesis are taking place before the eighth week of fertilization. Development of the brain by itself includes several major stages and lasts during the whole pregnancy. Major events in human brain development include primary neurulation, prosencephalic development, neuronal proliferation and migration, organization, and myelination [1]. The neuronal tube formation is already finished by the 20th day of fertilization [2], and the formation of the cortical plate takes place between 7 and 16 weeks of gestation though the cortex increases in thickness until neurogenesis is completed after midgestation. The total number of neurons in the central nervous system (CNS) reaches a maximum in the first 20–24 weeks of the antenatal period and remains relatively constant up to adulthood, only slightly decreasing in early postnatal period.

By the 9th week of gestation, ERα is detected in the proliferating zones and the cortical plate [2, 3]. In contrast to the expression patterns of ERα, ERβ is detected at 15 weeks of gestation in proliferating zones and at 16–17 weeks of gestation in the

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cortical plate. From the same period of time, both receptors are expressed in different subregions of the hippocampus [3]. In the rat pups, ERs are present in the developing as well as adult hippocampus [4, 5] with a peak in binding at postnatal day 4 declining to adult levels already by postnatal day 15 [6, 7]. Thus, ERα plays a role in early developmental processes, whereas ERβ might be more important for later events of corticogenesis [8]. Two pairs of internal carotid and vertebral arteries, connected by the circle of Willis, supply the brain with blood [2]. The internal carotid arteries develop quite early, by the 4th week of gestation, whereas by the 5th week of gestation, most of arteries are developed by forming a specific pattern [2]. At 16 weeks of gestation, the anterior, middle, and posterior cerebral arteries are already well established. In premature newborns between 22 and 30 weeks of gestation, the blood vessels of the germinal and periventricular zone and the perforating ventriculopetal vessels are particularly vulnerable to perinatal asphyxia [2], whereas between 30 and 34 weeks of gestation, the fetal white matter is vulnerable to hypoxic ischemic injury, and the injury leads to the formation of focal hemorrhagic lesions and periventricular leukomalacia (PVL) [often resulting in infarction (necrosis) and cavitation], respectively [2].

Myelination in the CNS is performed by oligodendrocytes and is a slow process which is a significative mark of the maturity of the CNS. Notably, in the brain stem, myelination starts at the 8th week of gestation though not completed until after birth, and the rate of myelin deposition is greatest during the first 2 postnatal years [9].

4.2 Estrogens, Estrogen Receptors (ERs), and the Brain

4.2.1 Estrogens

Successful maintenance of pregnancy requires the coordinated secretion of hormones. Indeed, placenta, feto-placental unit, and fetus become the main sources of estrogens: estrone (E1), estradiol (E2), estriol (E3), and estetrol (E4). Appearance of each estrogen in maternal plasma after 9 weeks of gestation coincides with main events of the brain development, pointing out the importance of estrogens in formation of brain morphology and its functionality. For example, we can easily follow the manifestation of unconjugated estradiol (E2) in maternal plasma by the 9th week of gestation and the detection of ERα in the proliferating zones and the cortical plate [2, 3], like that showing the importance of E2 in early corticogenesis. Plasma concentrations of E1, E2, E3, and E4 increase as human pregnancy progresses [10, 11] implicating importance of estrogens in fetal development during pregnancy in general as well as in parturition.

Some recent studies already have shown role of estrogens in (1) fetal neurogenesis, (2) prevention of neuronal cell death, (3) axonal sprouting, and (4) synaptic transmission [12, 13]. Others implicated estrogens as major players for fetal cerebral angiogenesis and cerebral blood flow maintenance due to (1) neurovascular sharing of major signaling pathways and the development of blood-brain barrier, (2) interaction between neurons and vessels mediated by Ca^{2+} ions released from astrocytes, (3) direct effects of estrogens on cerebral vessels [14–18], (4) decrease in

water permeability of the blood-brain barrier (BBB) [19], and (5) upregulation of vascular endothelial growth factor (VEGF) expression from neuronal cells [20].

VEGF is an angiogenic protein with neurotrophic and neuroprotective effects which stimulate neurogenesis in vitro and in vivo in the subventricular zone (SVZ) and the subgranular zone (SGZ) of the hippocampal dentate gyrus (DG) [21] and promotes proliferation of cortical neuron precursors by regulating E2F expression (the family of transcription factors, a key regulator of the cell cycle machinery) [22]. Usually neuronal VEGF expression correlates with angiogenesis in postnatal developing rat brain and might be upregulated by hypoxia [23], but it has importance in the developing brain as well. As it was already evidenced, loss of VEGF expression by CNS neurons impairs vascularization, curbs neuronal expansion, and results in neuronal apoptosis in the developing brain, pointing out that VEGF-induced blood vessel growth is essential for nervous tissue growth during embryonic development [24, 25].

According to different studies, general impact of estrogens on the CNS includes (1) neuromodulatory effect by affecting neuron excitability, synaptic plasticity, and neurotransmitter system; (2) neurotrophic effect by influencing glial morphology and functions, neurite outgrowth and sprouting, and cell viability; and (3) neuroprotective effect by exerting proneurogenic, antiexcitatory, antioxidative, antiapoptotic, and anti-inflammatory profile [26, 27].

Estrogen administration in animal models and clinical studies of Parkinson's and Alzheimer's diseases, ischemic stroke, spinal cord injury, and multiple sclerosis has already demonstrated neuroprotective effect [12, 28–32], suggesting the early protective effect of estrogens administered particularly in younger patients (50–62 years) [33] or in women soon after menopause [34]. Even short-term estrogen treatment increases dopamine transporters in the caudate putamen [35] and amyloid beta-protein (Aβ) uptake by microglia. It also prevents Aβ peptide formation by neurons [36, 37] and protects against loss of DA neurons [38, 39]. According to the "healthy cell bias of estrogen action hypothesis," if estrogens are administered too late in the disease, they are not protective [40, 41].

At cellular and molecular levels, estrogens might have different important actions: (1) increase of astrocyte ability to uptake glutamate and like that preventing neuronal loss due to glutamate toxicity [42, 43], (2) direct neuroprotective effect on mitochondria [44–48], (3) induction of expression of genes regulating cytoskeleton of neuron cells (e.g., neurofilaments, microtubule-associated proteins), and (4) increase of aerobic glycolysis, respiratory efficiency, ATP generation, and Ca^{2+} load tolerance leading to antioxidant defense [49].

4.2.2 Estrogen Receptors (ERs)

Estrogen actions are realized through specific estrogen receptors (ERs).

GPER is a membrane-bound receptor, though some research groups found it in the Golgi complex [50] or at the endoplasmic reticulum [51] or even intracellularly in some transfection experiments [52]. Estrogens may affect serotonin signaling in the hypothalamus [53] and dopamine efflux in PC-12 cells [54] or mechanical

hyperalgesia in nociceptive neurons of rat dorsal root ganglia [55] and control the energy homeostasis in the hypothalamus [56] by employing GPER.

ERs have specific regions, activation function 1 and 2 (AF1 and AF2), which are responsible for formation of initial transcriptional complexes. AF1 and AF2 are situated in the amino-terminal and carboxyl-terminal domains of the receptors, respectively [57]. These functions characterize most of nuclear receptors and correspond to two active domains which are responsible for recruitment of specific co-regulator proteins, and these proteins in turn might modulate transcriptional activity of ERα. The level of AF1 activity does not depend on the presence of ligand, whereas the activity of AF2 is ligand-dependent [57]. Inactive receptors are linked to the heat shock proteins, and binding of ligand to the ERs leads to dissociation of heat shock proteins from ERs followed by dimerization of ERs. ERs are forming homo- and heterodimers [58]. Zinc (Zn^{2+}) fingers of ligand-ERs complex bind to DNA at specific ERs elements (EREs) which are located in the regulatory regions of target genes and where they act as a hub for a large transcriptional complex including coactivators and corepressors resulting in gene transcription. EREs can alter transcription indirectly by interacting with other transcription factors (AP1, C/EBPβ, and SP1) [57, 58].

According to different studies, estrogen receptors (ERs), ERα, ERβ, and G protein-coupled estrogen receptor 1 (*GPER*, also known as G protein-coupled receptor 30 (GPR30)), may coexist in many brain areas, although their expression levels and distribution patterns are different and sometimes gender specific [59–63]. Like that in adult human brain, ERα, ERβ, and GPER coexist in the basal forebrain, hypothalamus, and hippocampus; ERα and ERβ are expressed in the prefrontal cortex, amygdala, locus coeruleus, and raphe nucleus; ERβ and GPER coexist in the thalamus, whereas only ERβ is expressed in the posterior cingulate [63]. During development ERα and ERβ display distinct chronology and distribution patterns that undergo dynamic changes in the course of corticogenesis as already discussed above.

In terms of subcellular localization, there is a difference between ER localization during development and in adults. During development ERα and ERβ are located in the cell nuclei, whereas in the adult human brain, the ERα staining is localized in both cell compartments (cytoplasm and nucleus), and ERβ has exclusively cytoplasmic localization [64, 65]. The ERα staining is clearly cytoplasmic in the pyramidal cells of Ammon's horn (CA) and in layer II of the entorhinal cortex, whereas it is more nuclear in the dentate gyrus (DG) and in layer V of the entorhinal cortex and temporal cortex [59, 64, 65]. Some recent investigations already have shown localization patterns of ERα, ERβ, and GPER in different neuronal cells pointing out involvement of these receptors in neurogenesis and myelination. In astrocytes and microglia, expression of ERα and ERβ is observed in the nuclei, whereas ERβ is expressed in the cytoplasm; nuclei of the axonal bodies express ERα and ERβ, whereas ERα, ERβ, and GPER are manifested in the cytoplasm; basal dendrites, axonal (initial) segments, and myelin sheaths are rich with ERβ [63]. Another question that became the main direction of research is subcellular localization of ERs in the dentate gyrus (DG) region of the hippocampus which is important for

neurogenesis and synaptic remodeling as well as neuroprotection and realization of cognitive function. In DG, a subset of GABAergic interneurons contains nuclear ERα, whereas granule cells, newly born cells, and some GABAergic interneurons contain cytosolic and plasma membrane-associated ERβ [64]. Dendritic spines, mostly originating from granule cells, contain ERα and ERβ. A few dendritic spines in the hilus of DG, originating from mossy cells, contain ERα and ERβ. Interestingly, some ERα-containing axon terminals are cholinergic, whereas some ERβ-containing terminals are monoaminergic. Astrocytes, mostly in the molecular layer, also contain ERα and ERβ [64].

In general, expression of ERα mRNA in the neonatal cortex, olfactory bulb and cerebellum suggests its role in the regulation of early postnatal differentiation and development of these brain areas by estrogens, since ERα is supposed closely related to cellular differentiation and sexual differentiation of developing brain [6].

Different studies already have demonstrated the presence of ERs in rat pial arteries and intracerebral blood vessels [65] and also proved expression of ERα in nuclei, membranes, and mitochondria of endothelial and vascular smooth muscles of cerebral arteries [19, 65]. Although ERβ was detected in immunoblots of cerebral artery lysates, the definitive role of ERβ is not fully understood [65], though ERα and ERβ may modulate each other's activity [66].

According to some studies, ER activation might offer neuroprotection, in part, through transcriptional mechanisms affecting the apoptotic cascade including BCl2, caspases, and Apaf-1 like that limiting cell death [67–70]. ERs can also directly activate signal transduction pathways involving MAP kinase resulting in neuroprotection that is receptor-mediated [71]. As it was shown in Parkinson's disease animal model of 6-hydroxydopamine (6-OHDA), estradiol can also act indirectly by activating the insulin-like growth factor-1 (IGF-1) receptor to protect against 6-OHDA-induced neuronal loss [72].

If the abovementioned effects of estrogens and ERs in the CNS were prominent mainly for E2, information on how E4 affects the CNS became available only few years ago based on studies performed by our research group.

4.2.3 Estetrol

Estetrol (E4) is a steroid hormone, discovered in 1965 by Egon Diczfalusy and co-workers [73]. Structurally, estetrol is an estrogenic steroid with four hydroxyl groups, explaining the acronym E4. Estetrol is produced in nature by the human fetal liver, since its synthesis requires two hydroxylases (15α- and 16α-hydroxylase) only expressed by the fetal liver during pregnancy. Substrates for E4 are estradiol (E2), requiring both 15- and 16-hydroxylation, and estriol (E3), requiring 15-hydroxylation only. Estetrol is an end product of steroid metabolism. There is no metabolism backward to E3, E2, or E1, and there are no active metabolites [74]. The chemical name of estetrol is estra-1,3,5(10)-trien-3,15a,16a,17b-tetrol, and it is known under CAS No. 15183-37-6. The molecular formula of E4 is

$C_{18}H_{24}O_4$, and it has a molecular weight of 304.38. Its physical appearance is that of a white to off-white solid. Estetrol has a melting point in the range of 240–245 °C [73]. Experience so far indicates that E4 is very stable, even under nonoptimal storage conditions. E4 might be slightly hygroscopic. Storage conditions of E4 should therefore be optimized to prevent moisture and water uptake. Both in pure water and in phosphate buffers, E4 is highly soluble. In water, the solubility amounted to 1.0 mg/ml. The octanol-water partition coefficient (Pow) is a measure of the lipophilic or hydrophilic properties of a compound and is expressed as the logarithm of Pow. The lipophilic and hydrophilic properties largely determine the passive gastrointestinal absorption, the distribution through the body, and the passive passage of the blood-brain barrier. In two sets of experiments, using different methods to determine the partition coefficient of E4, the observed log Pow values were 1.470 and 1.695 [73, 74]. This means that concentrations in the octanol phase were about 30–50 times higher compared to those in the water phase. A log Pow of about 2.0 is considered optimal to allow passage through the blood-brain barrier [74].

E4 is found in maternal urine as early as 9 weeks of gestation, increasing substantially as pregnancy progresses [74, 75]. Estetrol produces a number of biological changes in the rodent uterus, such as weight increase, progesterone receptor stimulation, enzyme induction, and histological and ultrastructural changes. From a teleological viewpoint, it seems likely that an estrogenic steroid produced in such significant quantities by the male and female human fetal liver during pregnancy is safe and has physiological significance. As it was concluded, genomic clinical effects of E4 will most likely occur through the estrogen receptors. E4 has a moderate affinity for human ERα and ERβ, with Ki values of 4.9 + 0.567 nmol/l and 19 + 1 nmol/l, respectively, demonstrating a four- to fivefold preference for the ERα (lower Ki value) [76]. Estetrol has high selectivity for the estrogen receptors. Binding to the glucocorticoid, progesterone, and testosterone receptors was only 11–15% at a concentration of 10 mmol/l, and further profiling of E4 in a set of 124 receptors and enzymes demonstrated inactivity toward 123 molecular targets. The single target showing interaction with E4 was the adrenergic α1β receptor (weak binding) [76]. It is concluded that genomic clinical effects of E4 will most likely occur through the estrogen receptors. The high selectivity of E4 suggests a low risk of unexpected side effects [77, 78]. E4 could be a safe and efficacious candidate for the treatment of early brain damage in newborn. The use of E2 unlike the use of E4 might have diverse effects on inflammation and immune responses [79], cardiovascular complications, venous thrombosis, and stroke [80–82], even the initiation/progression of several endocrine-related cancers (e.g., breast, prostate, ovarian, and endometrial cancer) [83].

Main properties of E4 are as follows: (1) slow metabolism and the long half-life; (2) strong antioxidant properties; (3) no binding to sex hormone-binding globulins (SHBG), suggesting that E4 may not influence the plasma levels of SHBG; and (4) log Pow index about 1.470–1.695 which is enough for any compound to pass the blood-brain barrier [75–77]. These properties of E4 might be important to assume the possible neuroprotective actions of E4.

4.3 Neonatal Hypoxic-Ischemic Encephalopathy

Neonatal encephalopathy (NE) is a clinically defined syndrome of disturbed neurologic function in the earliest days of life in an infant born at or beyond 35 weeks of gestation, manifested by a subnormal level of consciousness or seizures and often accompanied by difficulty with initiating and maintaining respiration and depression of tone and reflexes [84]. Neonatal hypoxic-ischemic encephalopathy is associated with increased lethality and long-term morbidity. Mortality and the neurodevelopmental outcomes in infants with moderate and severe HIE are as follows: 23–27% of infants die prior to discharge from the neonatal IC unit (NICU), whereas 37–38% die at follow-up 18–22 months later. The neurodevelopmental outcome at 18 months includes mental and psychomotor development retardation, cerebral palsy, epilepsy, blindness, and hearing impairment [85, 86]. Usually neonates with HIE have different complications manifested with the different degree of severity as follows: mental development index (MDI) <70 (39%), psychomotor development index (PDI) <70 (35–41%), disabling cerebral palsy (30%), epilepsy (16%), blindness (14–17%), and severe hearing impairment (6%) [85, 86], and HIE is the fifth largest cause of death of children before age 5 [87–89].

Perinatal asphyxia or birth asphyxia, more appropriately known as neonatal hypoxic-ischemic encephalopathy (HIE) or hypoxic and ischemic brain injury in the newborn, is characterized by clinical and laboratory evidence of acute or subacute brain injury (encephalopathy) due to intrapartum or late antepartum brain hypoxia and ischemia [90, 91]. A common but crucial problem is the inability to time the onset, duration, magnitude, and single or repetitive nature of the exact insult that causes brain injury resulting in neonatal encephalopathy. The uncertain timing and etiology of brain injury in most cases of neonatal encephalopathy also fuel birth injury malpractice litigation. It is usually unknown whether the ultimate brain injury is caused by the events only around delivery or by cumulative insults throughout pregnancy [84].

Health factors that influence the risk of neonatal encephalopathy include maternal diseases, multiple pregnancy, gestational age at delivery, malformations within or outside the nervous system, intrauterine growth restriction, congenital infections, intrapartum hypoxic-ischemic events, metabolic problems, and stroke [84–86]. Type and timing of contributing factors that are consistent with an acute peripartum or intrapartum events include sentinel hypoxic or ischemic event occurring immediately before or during labor and delivery (a ruptured uterus, severe abruption placentae, umbilical cord prolapsed, amniotic fluid embolus with coincident severe and prolonged maternal hypotension and hypoxemia, maternal cardiovascular collapse, fetal exsanguinations from either vasa previa or massive feto-maternal hemorrhage) [84]. More precisely, there are several risk factors associated with the development of perinatal HIE: preconceptual (e.g., diabetes mellitus type 1, thyroid disease, fertility treatment, nulliparity, advanced maternal age), antepartum (severe preeclampsia, placental abruption, multiple pregnancy, antepartum hemorrhage, fetal growth restriction), and intrapartum (e.g., breech and malpresentation, cord prolapse, caesarean section, maternal pyrexia, induction) [84, 91, 92]. Given the history of the

understanding of NE and HIE, it's not surprising that HIE has been most commonly studied in vitro and in vivo. Consequently we will focus on the understanding of the cellular mechanisms of HIE because this is the pathway to NE that has been best studied [84].

The principal pathogenetic mechanism underlying most of the neuropathological conditions leading to hypoxia-ischemia is a failure of compensatory mechanisms and impaired cerebral blood flow (CBF). At the cellular level, hypoxia-ischemia initially causes energy failure, reperfusion and oxidative and nitrosative stress (immediate phase), and then the loss of mitochondrial function and caspase activation (delayed phase) [88, 93]. Step by step, the primary energy failure is accompanied by glutamate-mediated excitotoxicity. Excitotoxic cellular injury occurs via excess activation of glutamate receptors, which leads to necrotic cell death within 6 h after insult and is more prominent within 1.5 h after insult. There are four receptor types for glutamate, but the *N*-methyl-D-aspartate (NMDA) receptors are the most avid and physiologically active. The channels activated by NMDA receptors are voltage-dependent and calcium-permeable. Their activation causes neuron depolarization. Repeated depolarization of a neuron by unregulated glutamate release results in accumulation of intracellular calcium. Consequently, an increase of intracellular calcium sets off additional pathologic cascades [88, 93] including oxidative stress and interaction with nitric oxide pathway to produce reactive nitrogen species—peroxynitrites leading to peroxynitrite-induced neurotoxicity, lipid peroxidation, mitochondrial damage and remodeling, depletion of antioxidant reserve, and DNA damage [94]. Between 6 and 72 h after insult, development of mitochondrial dysfunction leads to caspase activation and to the apoptotic cell death which is more prominent during the first 6–8 h after insult [88]. This is the point of "no return." Inflammatory mediators (cytokines and chemokines) have been implicated in the pathogenesis of hypoxic-ischemic encephalopathy and may represent a final common pathway of brain injury. As it was shown, NF-kB activation in neurons could provide survival, whereas activation in glial cells enhances neuronal cell death [95].

Assuming that hypoxic-ischemic encephalopathy represents the clinical condition affecting mostly the brain, brain lesions have been reported in many studies. Pial arteriolar vasodilatation is a constant finding in the brain of asphyxiated newborns. It is simply evidenced at panoramic view, and it is mainly related to the loss of microvascular reactivity in cerebral vessels [95, 96]. Endothelial damage represents probably the most important change in the brain of asphyxiated newborns. All the endothelial lesions previously reported may be encountered at the histological examination of brain samples [97]. Endothelial swelling represents a peculiar feature in the small intracerebral vessels. Given the narrow lumen of the intravascular capillaries, endothelial swelling may lead to the occlusion of the vascular lumen, leading to the block of the intracerebral circulation, aggravating brain hypoxia. The endothelial damage is followed by the dysfunction of the neurovascular unit that contributes to subsequent neuronal cell death [97, 98]. Neuronal cell death represents a major pathological finding in the interpretation of the severity of the hypoxic encephalopathy. Apoptosis is the most frequent type of cell death

occurring in the brain of asphyxiated newborns. At histology, affected neurons show shrinkage, increased eosinophilia of the cytoplasm, nuclear pyknosis, and karyorrhexis, ending with the formation of roundish eosinophilic globules that appear intermingled with preserved neurons [97]. Neuronal apoptosis may be encountered, in the clinical setting of asphyxia, in all the cerebral regions. In our experience, neurons of the brain stem, basal nuclei, and cerebellum appear as the most frequently affected by apoptosis, often in association with apoptosis of the cerebral cortical neurons. Recently, the increased expression of pro-apoptotic proteins—including BAX, cytoplasmic cytochrome C and caspase-3—has been reported in the cortex and thalamus of the brain of mice affected by birth hypoxia [97, 99], suggesting the use of these antibodies in cases in which histology could not clearly evidence the typical features of neuronal cell death. The hippocampus should be always sampled for histological studies, given the frequent functional compromise of this brain region in newborns affected by asphyxia, particularly in female infants [96, 100]. In a recent study, all 16 full-term asphyxiated infants displayed neuronal cell damage and glial reactivity in the hippocampus [96, 101]. If we are talking about the patterns of neonatal HIE at term, there are five patterns of brain injury identified by imaging studies in neonates with this pathological condition: pattern I, basal ganglia and thalami lesions associated with severe white matter damage; pattern II, basal ganglia and thalami lesions with mild or moderate white matter changes; pattern III, isolated thalamic injury; pattern IV, moderate white matter damage only; and pattern V, mild white matter changes or normal findings. Usually infants with patterns III and IV had developmental delay and diplegic cerebral palsy, respectively, and pattern V is associated with normal outcomes [102]. Thus, the basal ganglia and thalami lesions are the imaging signature in term neonates exposed to hypoxic-ischemic sentinel events, and in general, patterns of central gray matter and secondary white matter injury are associated with higher risks of severe morbidity and death [102].

Clinical manifestation of neonatal encephalopathy varies depending on encephalopathy severity.

Neonates with suspected encephalopathy are classified according to the Sarnat staging system, which evaluates the level of consciousness, muscle tone, tendon reflexes, complex reflexes, and autonomic function and classifies HIE into the following three categories: stage I (mild), stage II (moderate), and stage III (severe) [103, 104].

*Stage I: Mild Encephalopathy*Muscle tone may be slightly increased, and deep tendon reflexes may be brisk during the first few days. Transient behavioral abnormalities, such as poor feeding, irritability, or excessive crying or sleepiness (typically in an alternating pattern), may be observed [88, 103, 104].

*Stage II: Moderate Encephalopathy*The infant is lethargic, with significant hypotonia and diminished deep tendon reflexes. The grasping, Moro, and sucking reflexes may be sluggish or absent. The infant may experience occasional periods of apnea. Seizures typically occur early within the first 24 h after birth [103]. Full recovery within 1–2 weeks is possible and is associated with a better long-term outcome. An initial period of well-being of mild encephalopathy may be followed by sudden

deterioration, suggesting ongoing brain cell dysfunction, injury, and death; during this period, seizure intensity might increase [88, 103, 104].

*Stage III: Severe Encephalopathy*Stupor or coma is typical. The infant may not respond to any physical stimulus. Breathing may be irregular, and the infant often requires ventilatory support. Generalized hypotonia and depressed deep tendon reflexes are common. Neonatal reflexes (e.g., sucking, swallowing, grasping, Moro) are absent [88, 103, 104]. Disturbances of ocular motion, such as a skewed deviation of the eyes, nystagmus, bobbing, and loss of "doll's eye" (i.e., conjugate) movements may be revealed by cranial nerve examination [88]. Pupils may be dilated, fixed, or poorly reactive to light. Seizures are delayed, can be severe, and may be initially resistant to conventional treatments. The seizures are usually generalized, and their frequency may increase during the 24–48 h after onset, correlating with the phase of reperfusion injury. As the injury progresses, seizures subside, and the EEG becomes isoelectric or shows a burst suppression pattern. At that time, wakefulness may deteriorate further, and the fontanelle may bulge, suggesting increasing cerebral edema [88].

Irregularities of heart rate and blood pressure (BP) are common during the period of reperfusion injury, as is death from cardiorespiratory failure. Multiple organ dysfunction also presents [88]. Multi-organ systems involvement is a hallmark of NE associated with perinatal asphyxia [88]. Organs involved following a hypoxic-ischemic events include the heart (43–78%) with reduced myocardial contractility, severe hypotension, passive cardiac dilatation, and tricuspid regurgitation; lungs (71–86%) with severe pulmonary hypertension requiring assisted ventilation; kidneys (46–72%) with renal failure presenting as oliguria and, during recovery, as high-output tubular failure, leading to significant water and electrolyte imbalances; liver (80–85%) with elevated liver function test results, hyperammonemia, and coagulopathy; and gastrointestinal system with poor peristalsis and delayed gastric emptying, and necrotizing enterocolitis is rare, and intestinal injuries may not be apparent in the first few days of life or until feeds are initiated; hematologic disturbances (32–54%) include increased nucleated red blood cells (RBCs), neutropenia or neutrophilia, thrombocytopenia, and coagulopathy [88]. Severely depressed respiratory and cardiac functions and signs of brain stem compression suggest a life-threatening rupture of the vein of Galen (i.e., great cerebral vein) with a hematoma in the posterior cranial fossa.

NE is often reported to be the most frequent cause of neonatal seizures [88]. Large, unilateral infarcts occur with neonatal seizures in as many as 80% of patients. Infants with multiple or diffuse lesions and cerebral venous infarcts often have multifocal or migratory seizures observed even during physical examination [88].

A single valuable test for the diagnosis of HIE does not exist. It is important to assess the neonate at birth for detection of signs consistent with an acute peripartum or intrapartum event and the designation of perinatal asphyxia severe enough to result in acute neurologic injury [84]. The following parameters should be taken into consideration: (1) Apgar score of less than 5 at 5 and 10 min after birth; (2) umbilical artery pH less than 7.0, or base deficit greater than or equal to 12 mmol/l, or both; (3) neonatal neurologic sequelae (e.g., seizures, coma, hypotonia); and (4) multiple organ involvement (e.g., renal injury, hepatic injury, hematologic abnormalities, cardiac dysfunction, metabolic derangements, and gastrointestinal injury, or a

combination of them) [84, 88]. Although the presence of organ dysfunction increases the risk of HIE in the setting of neonatal encephalopathy, the severity of brain injury seen on neuroimaging does not always correlate with the degree of injury to other organ systems [84]. Nowadays, magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS), as the most sensitive neuroimaging modalities, are extensively used for the monitoring and evaluation of neonates with NE [84]. Distinct patterns of neuroimaging abnormalities, including deep nuclear gray matter or watershed cortical injury, are recognized in hypoxic-ischemic cerebral injury and have prognostic value for predicting later neurodevelopmental impairments. Early MRI obtained between 24 and 96 h of life may be more sensitive for the delineation of the timing of perinatal cerebral injury, whereas an MRI undertaken optimally at 10 days of life (with an acceptable window between 7 and 21 days of life) will best delineate the full extent of cerebral injury [84]. Use of electroencephalography (EEG) might have some limitations due to hypothermia: it may depress the amplitude-integrated EEG (aEEG) and thus limit the early predictive ability of aEEG. Improvement in aEEG tracings may be delayed until the patient undergoes rewarming and is no longer sedated [105]. Some recent investigations showed that S100B protein is a good indicator of brain damage [106]. Furthermore, the serum GFAP levels during the first week of life were increased in neonates with HIE and were predictive of brain injury on MRI. Biomarkers such as S100B and glial fibrillary acidic protein (GFAP) could help triage neonates with HIE to treatment, measure treatment efficacy, and provide prognostic information [106, 107].

According to the contemporary treatment strategy for HIE, initial resuscitation and stabilization are followed by the following steps: (1) neuroprotective strategy, (2) support of adequate ventilation and perfusion, (3) careful fluid management, (4) avoidance of hypo- and hyperglycemia, (5) avoidance of hypotension [a mean blood pressure (BP) above 35–40 mmHg is necessary to avoid decreased cerebral perfusion], and (6) treatment of seizures [108, 109].

At present, hypothermia therapy (HT) is considered as the best neuroprotective strategy for mild to moderate HIE. Mild hypothermia in the range of 33.5–35.0 °C is used. The two types of treatment are used: whole-body hypothermia and selective head cooling. Some basic studies showed far greater histological and electrophysiological protection if hypothermia was initiated within 1.5 h than if it was started 5.5 h after the cerebral insult [110]. According to recent trials, neonates undergoing earlier cooling therapy (within 180 min of birth) had better outcomes compared with those who underwent the therapy later (180–360 min after birth) [111]. The rate of death or severe disability in infants with HIE is decreased from 60% to 46% after cooling [112].

Although cooling is safe, it results in some adverse effects which include a slightly lower baseline heart rate, a marginally significant increase in the need for blood pressure support, and a platelet count below 150×10^9/l [113]. In general, lowering the core temperature can impact hemodynamic status, respiratory physiology, fluid and electrolyte balance, and hematologic factors. In addition, pharmacokinetics and pharmacodynamics of a number of drugs commonly used in asphyxiated neonates are affected by hypothermia. Careful attention to physiologic parameters, laboratory tests, and drug dosing is essential to assure optimum outcomes for

neonates undergoing hypothermia therapy [114]. There are some risks associated with return from hypothermia to normothermia as follows: (1) apnea, (2) the risk for seizures (increases due to rewarming leading to peripheral vasodilatation and the intravascular blood volume increase) [115], (3) hypotension (may occur if the vascular bed is underfilled), and (4) alteration in the cardiac function (as a result of the initial hypoxic event may play a contributing role) [110].

Importance of searching for new safe neuroprotective strategy alone or in combination with hypothermia therapy became crucial. We paid our attention to already described specific properties of E4 which were important to come up with the hypothesis that E4 might have neuroprotective effect.

4.4 Estetrol as a New Drug to Treat Neonatal HIE

4.4.1 Estetrol Attenuates Neonatal HIE

Our primary goal was to define effects of E4 in primary hippocampal cell cultures. In vitro experiments with primary hippocampal neuronal cell cultures showed impressive antioxidative and cell survival effects of different doses of E4 (notably, 650 µl, 3.25 mM, and 6.5 mM) used before or after induction of oxidative stress (Fig. 4.1a, b, c, and d, respectively) [116]. More precisely, E4 at a dose of 3.25 and 6.5 mM significantly downregulated the LDH activity in both sets of experiments (Fig. 4.2a, c), showing the dose-dependent differences at higher

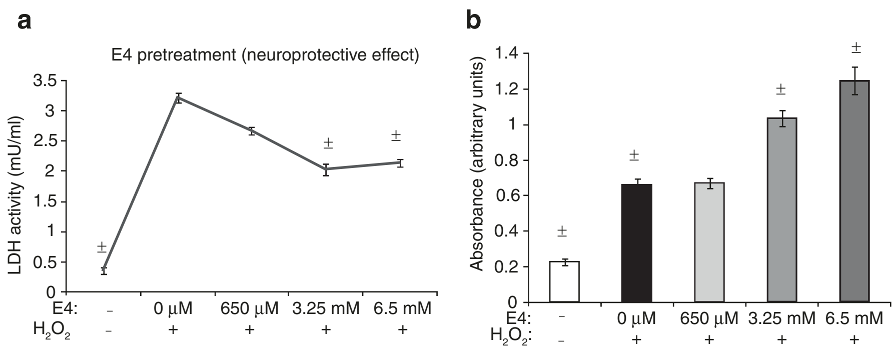

Fig. 4.1 Effect of E4 on LDH activity and the cell viability in primary hippocampal neuronal cultures subjected to the H_2O_2-induced oxidative stress. (**a**, **b**) E4 pretreatment. Primary hippocampal cell cultures were treated either with vehicle or with 650 µM, 3.25 mM, and 6.5 mM of E4 (1 h) before induction of oxidative stress by 100 µM of H_2O_2 (30 min). (**a**) LDH activity in untreated, H_2O_2-treated, and E4-pretreated cell cultures; (**b**) cell viability in untreated, H_2O_2-treated, and E4-pretreated groups; (**c**, **d**) E4 treatment. Primary hippocampal cell cultures were treated with 650 µM, 3.25 mM, and 6.5 mM of E4 (1 h) after induction of oxidative stress by 100 µM of H_2O_2 (30 min). (**c**) LDH activity in untreated, treated by H_2O_2, and E4-treated cell cultures; (**d**) cell viability in untreated cells as well as cells treated by H_2O_2 and different concentrations of E4. All measurements are expressed as mean ± SEM. $^*p < 0.05$. Reproduced from *Experimental Neurology* (Tskitishvili et al., 2014, 261:298–307)

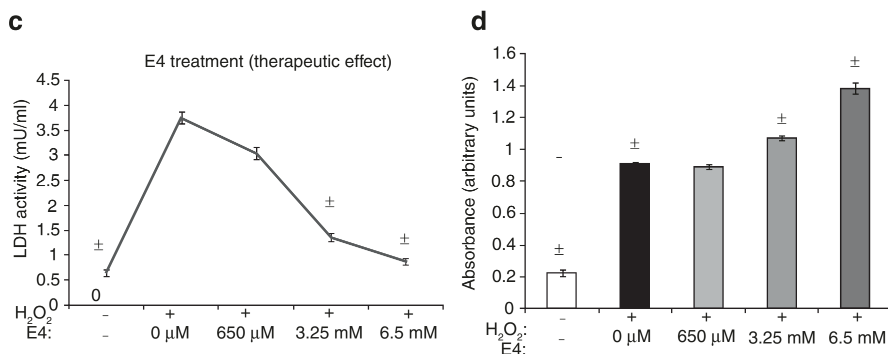

Fig. 4.1 (continued)

doses of E4 (3.25 and 6.5 mM) after induction of oxidative stress (Fig. 4.2c), whereas the same doses of E4 demonstrated significant upregulation of cell proliferation (Fig. 4.2b, d) [116].

Also our aim was to study the effect of E4 on brain damage in vivo. In vivo studies of hypoxic-ischemic brain injury were performed according to two protocols in newborn rat pups. A preventive model was tested with different doses of E4 injected to the rat pups before ischemic injury. E4 was administered intraperitoneally from postnatal day 4 (P4) to postnatal day 7 (P7). Subsequently, the left common carotid artery was ligated and cut. After recovery, the pups were exposed to low oxygen tension (8%) for 30 minutes, in a closed chamber. The therapeutic model consisted, first in the ligation at P7, of the common carotid artery and exposure to 8% oxygen for 30 minutes, to induce an hypoxic-ischemic insult (HI). The various doses of E4 were injected upon retrieval of the rat pups from the hypoxia chamber. All manipulations were performed at 37 °C. The sham group was also operated, but the carotid artery was neither ligated nor cut and the pups were not exposed to hypoxia. Rat pups recovered with their dams and reared normally until being sacrificed at P14 [116–118].

E4 did affect neither body weight nor brain weight or body temperature of the rat pups in both sets of experiments when E4 was applied before or after induction of HI events [116]. Histochemical studies of brain coronal sections revealed massive damage of the hippocampus and the cortex at the left (damaged) side of the brain (Fig. 4.2a2, b2) along with dilation of the central and the lateral ventricles in the vehicle-pretreated/treated groups [116].

Usually a good predictor of the neuroprotective activity of the compound is the counting of intact neuronal cells per visual field. Obviously, among E4-pretreated/treated groups, the hippocampus and the cortex were more preserved in sham, 5, 10, and 50 mg/kg/day E4 groups compared to the vehicle group in each region (Fig. 4.2a(1, 4–6), b(1, 4–6)). Intact cell counting was significantly different between the groups pretreated by E4 before induction of experimental hypoxic-ischemic brain injury, in the hippocampal and the cortical regions: vehicle and 5 mg/kg/day E4 groups (DG); vehicle and 5 and 10 mg/kg/day groups or sham and 1 and 50 mg/kg/day E4 groups (SGZ); sham and 1 and 10 mg/kg/day E4 groups (CA1); vehicle and sham, 50 mg/kg/day, or sham and 1 mg/kg/day E4 groups; whereas in the

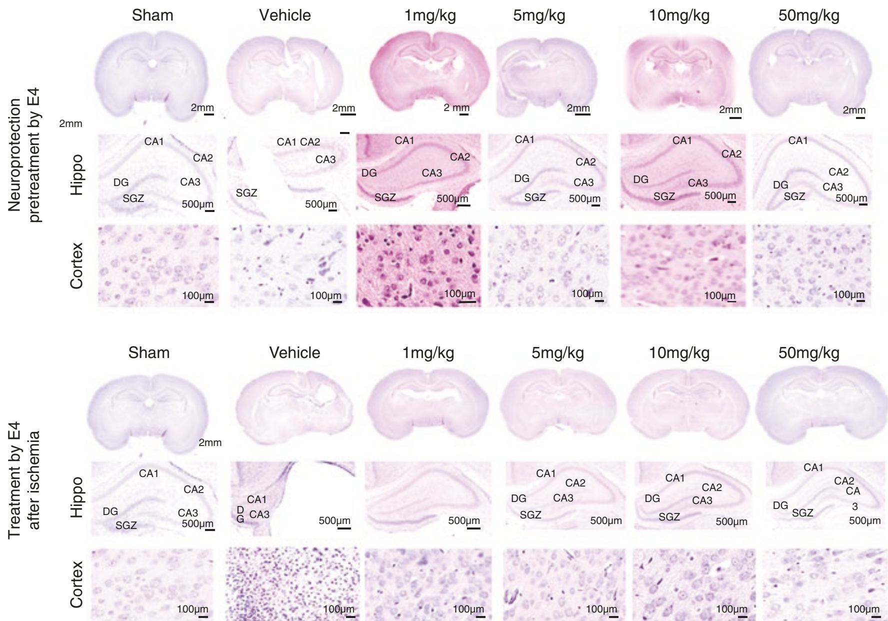

Fig. 4.2 Hematoxylin-eosin staining of coronal brain sections in the rat pups pretreated or treated with E4. Brains of the rat pups were removed upon sacrifice at P14; paraformaldehyde-fixed and paraffin-embedded samples were processed for sectioning at the hippocampus region and hematoxylin-eosin staining. Brain coronal sections of the rat pups (scale bar, 2 mm) with hippocampus region (scale bar, 500 μm) and cortex (scale bar, 100 μm) are shown. E4 pretreatment: sham, vehicle, 1 mg E4/kg/day, 5 mg E4/kg/day, 10 mg E4/kg/day, and 50 mg E4/kg/day. E4 treatment: sham, vehicle, 1 mg E4/kg/day, 5 mg E4/kg/day, 10 mg E4/kg/day, and 50 mg E4/kg/day groups. Reproduced from *Experimental Neurology* (Tskitishvili et al., 2014, 261:298–307)

cortex, the intact cell counting was significantly higher in 50 mg/kg/day E4-pretreated group (Table 4.1) like that proving impressive neuroprotective effect of E4 in the brain [116].

E4 treatment after induction of hypoxic-ischemic brain injury in newborn rat pups resulted in significant difference of intact cell counting in the hippocampal and the cortical regions between the groups as follows: vehicle and 1 mg/kg/day, sham and 5, 10, and 50 mg/kg/day E4 groups (CA1); vehicle and 10 mg/kg/day or sham and 1, 5, 10 mg/kg/day, and 50 mg/kg/day E4 groups (CA2/CA3); whereas in the cortex, the intact cell counting was significantly different between the vehicle and 1 mg/kg/day or sham and 5, 10, and 50 mg/kg/day E4 groups (Table 4.1) like that proving the importance of therapeutic effect of E4 [116].

Contemporary basic and translational research studies in the field of neurology frequently employ different technics and methodologies from proof of concept to

Table 4.1 E4 retreatment is incorrect please correct to E4 pretreatment

	E4 pretreatment						E4 treatment					
	Sham	Vehicle	1 mg/kg/day	5 mg/kg/day	10 mg/kg/day	50 mg/kg/day	Sham	Vehicle	1 mg/kg/day	5 mg/kg/day	10 mg/kg/day	50 mg/kg/day
DG	161 ± 7	85 ± 6[a]	110 ± 7	121 ± 8	114 ± 8	110 ± 9	161 ± 7	88 ± 19[b]	113 ± 9	126 ± 10	137 ± 16	137 ± 17
SGZ	61 ± 5[d]	24 ± 3[c]	35 ± 3	43 ± 4	48 ± 5	31 ± 4	61 ± 5	28 ± 7[e]	35 ± 3	46 ± 8	45 ± 7	50 ± 7
CA1	71 ± 5[f]	52 ± 4	43 ± 2	48 ± 3	57 ± 4	52 ± 4	71 ± 5[h]	28 ± 7[g]	51 ± 2	45 ± 3	46 ± 3	47 ± 5
CA2/CA3	57 ± 6[j]	29 ± 3[i]	33 ± 3	48 ± 6	39 ± 3	53 ± 5	57 ± 6[l]	14 ± 3[k]	34 ± 4	34 ± 4	35 ± 3	30 ± 3
Cortex	69 ± 5	52 ± 5[m]	52 ± 3	71 ± 5	55 ± 3	76 ± 4	69 ± 5[o]	23 ± 4[n]	42 ± 5	57 ± 7	57 ± 6	54 ± 3

Significant differences were observed:
In the DG region: [a]vehicle vs. sham, 5 mg/kg/day E4-pretreated groups; [b]vehicle vs. sham in E4-treated groups
In the SGZ: [c]vehicle vs. sham, 5, and 10 mg/kg/day E4; [d]sham vs. 1 and 50 mg/kg/day E4-pretreated groups; [e]vehicle vs. sham in E4-treated groups
In the CA1 region: [f]sham vs. 1 and 10 mg/kg/day E4-pretreated groups; [g]vehicle vs. sham, 1 mg/kg/day E4; [h]sham vs. 5, 10, and 50 mg/kg/day E4-treated groups
In the CA2/CA3 region: [i]vehicle vs. sham and 50 mg/kg/day E4; [j]sham vs. 1 mg/kg/day E4-pretreated groups; [k]vehicle vs. sham, 10 mg/kg/day; [l]sham vs. 1, 5, 10, and 50 mg/kg/day E4-treated groups
In the cortex: [m]vehicle vs. 50 mg/kg/day E4-pretreated groups; [n]vehicle vs. sham, 5, 10, and 50 mg/kg/day E4; [o]sham vs. 1 mg/kg/day E4-treated groups
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fully operational status. Among different issues studies of the gray and white matter and neuro- and cerebro-angiogenesis by using specific markers have paramount importance.

As it was already demonstrated earlier, microtubule-associated protein 2 (MAP-2), as a cytoskeleton protein, has its value in the growth, differentiation, and plasticity of neurons, playing key roles in neuronal responses to growth factors, neurotransmitters, synaptic activity, and neurotoxins [119]. It is frequently used as a marker of early gray matter loss in immunohistochemistry studies.

As shown in Fig. 4.3A, B in both models of in vivo hypoxic-ischemic brain injury in the vehicle groups, MAP-2 staining was negative in the hippocampus of the left hemisphere extended to the cortex (Fig. 4.3A(b), B(b)). Further calculations demonstrated that the ratio of the MAP-2-positive area was significantly higher not only in the sham-operated animals (Fig. 4.3A(a), B(a)) but in animals pretreated/treated by different concentrations of E4 (Fig. 4.3A(c)–(f), B(c)–(f)) compared to

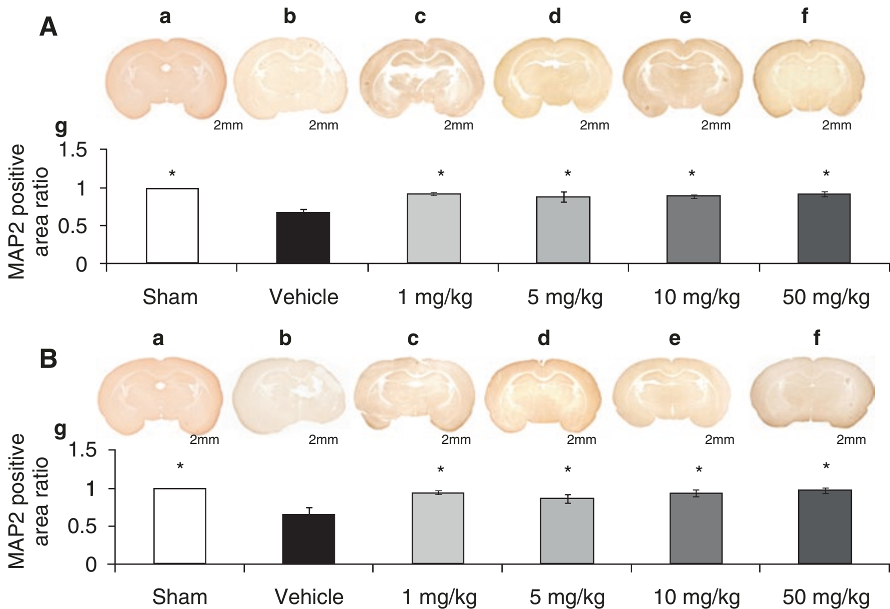

Fig. 4.3 MAP-2 staining of brain coronal sections in the rat pups pretreated or treated with E4 The sections were processed for immunohistochemical detection of neuronal cytoskeletal disruption. (**A**) E4 pretreatment: sham group (a), vehicle-treated group (b), 1 mg/kg/day E4 (c), 5 mg/kg/day E4 (d), 10 mg/kg/day E4 (e), and 50 mg/kg/day E4 (f) groups (g); (**B**) E4 treatment: sham group (a), vehicle-treated group (b), 1 mg/kg/day E4 (c), 5 mg/kg/day E4 (d), 10 mg/kg/day E4 (e), and 50 mg/kg/day E4 (f) groups. (g) The ratio of the MAP-2 positive areas was calculated as the MAP-2 positive area of the ipsilateral hemisphere divided by the MAP-2 positive area of the contralateral hemisphere. Ten samples from each group were analyzed. The ratio of the MAP-2 positive area in sham-operated animal group was considered by default as 1.0. All measurements are expressed as mean ± SEM. $*p < 0.05$. Reproduced from *Experimental Neurology* (Tskitishvili et al., 2014, 261:298–307)

the vehicle groups in both models (Fig. 4.4a(g), b(g)). Thus, E4 can preserve the early gray matter loss when administered before or after induction of experimental hypoxic-ischemic brain injury [116].

Definitely, for us, it was important to show the possible promyelinating activity of E4. Myelin basic protein (MBP), as a marker of white matter damage/demyelination, is frequently used in studies connected to brain damage [120].

There was a loss of MBP staining at the damaged left side (Figs. 4.4a and 4.5a) which was more prominent in main white matter regions [the subcortical region and the cingulum (Figs. 4.4b and 4.5b)]. Promyelinating effect of E4 was significantly upregulated in groups treated by 5 and 50 mg/kg/day E4 before induction of hypoxia-ischemia (Fig. 4.4c), whereas all the groups treated by different doses of E4 after induction of experimental brain injury had significantly higher MBP-positive area OD ratio along with the sham group (Fig. 4.5c) [118]. Significant positive correlation was observed between the myelination and the brain weights ($r = 0.707$, $p = 0.0198$) in the vehicle group of the neuroprotective in vivo model [118].

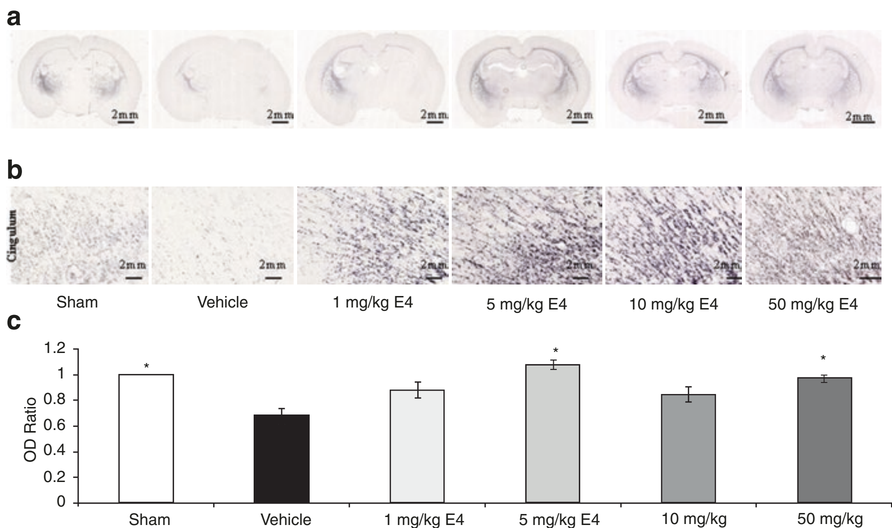

Fig. 4.4 Myelin basic protein (MBP) staining of brain coronal sections in rat pups pretreated with estetrol. (**a**) MBP staining of brain coronal sections (scale bar, 2 mm) is shown. (**b**) MBP staining of cingulum of the left hemisphere is shown (scale bar, 2 mm). (**c**) The ratio of the MBP-positive areas OD ratio was calculated as the MBP-positive area OD of the ipsilateral hemisphere divided by the MBP-positive area OD of the contralateral hemisphere. Ten samples from each study group were analyzed. The ratio of the MBP-positive area OD in the sham group was considered by default as 1.0. The MBP-positive area OD ratio was significantly higher in sham-operated animals and the 5 and 50 mg/kg/day E4-pretreated groups compared to the vehicle group. All measurements are expressed as mean ± SEM. *$p < 0.05$. Reproduced from J Endocrinol (Tskitishvili et al., 2017, 232(1):85–95)

Therapeutic effect

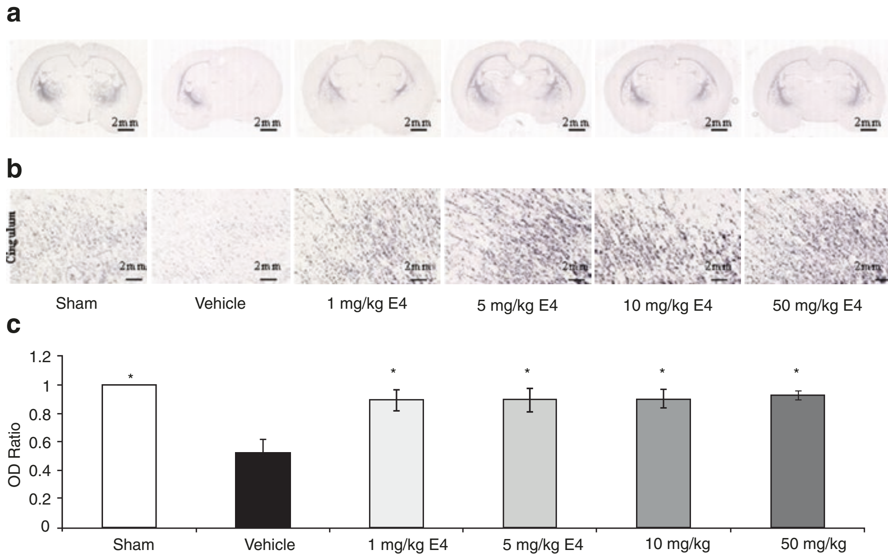

Fig. 4.5 Myelin basic protein (MBP) staining of brain coronal sections in rat pups treated with estetrol. (**a**) MBP staining of brain coronal sections (scale bar, 2 mm) is shown. (**b**) MBP staining of cingulum of the left hemisphere is shown (scale bar, 2 mm). (**c**) The ratio of the MBP-positive areas OD ratio was calculated as the MBP-positive area OD of the ipsilateral hemisphere divided by the MBP-positive area OD of the contralateral hemisphere. Ten samples from each study group were analyzed. The ratio of the MBP-positive area OD in the sham group was considered by default as 1.0. The MBP-positive area OD ratio was significantly higher in sham-operated animals and the 1, 5, 10, and 50 mg/kg/day E4-treated groups compared to the vehicle group. All measurements are expressed as mean ± SEM. *$p < 0.05$. Reproduced from J Endocrinol (Tskitishvili et al., 2017, 232(1):85–95)

Next step in studies of E4 and its neurological effects in brain was connected to the possible neurogenic- and cerebro-angiogenic effects of E4. Doublecortin (DCX), as a marker of neurogenesis, and VEGF as a marker for angiogenesis were used (Fig. 4.6). Obviously, pretreatment/treatment with different doses of E4 upregulated expression of the abovementioned markers in different regions of the brain and in different manner in all the study groups, showing co-localization of both markers in the cortical region of the 10 mg/kg/day E4-treated group (Fig. 4.4) [116]. In general, E4 pretreatment caused a significant upregulation of neurogenesis and cerebro-angiogenesis in the DG region, with 10 mg/kg for doublecortin and with all doses of E4 for VEGF (Table 4.2); in the CA1 region, in 5 mg/kg/day, 10 mg/kg/day, and 10 mg/kg/day E4 groups, respectively (Table 4.2); in the CA2/CA3 region, in 5 and 10 mg/kg/day E4 groups, respectively (Table 4.2); and in the cortex, in 10 mg/kg/day E4 (Fig. 4.4). Treatment by E4 after hypoxic-ischemic insult (Table 4.2) showed significant upregulation of neuro- and cerebro-angiogenesis in the CA1 region—in 10 and 50 mg/kg/day and in 5, 10, and 50 mg/kg/day E4 groups,

Table 4.2 Percentage of the DCX and VEGF positively stained cells in E4-pretreated/treated groups

			E4 pretreatment					E4 treatment			
	Sham	Vehicle	1 mg/kg/day	5 mg/kg/day	10 mg/kg/day	50 mg/kg/day	Vehicle	1 mg/kg/day	5 mg/kg/day	10 mg/kg/day	50 mg/kg/day
DG/DCX	43.7 ± 2.7	32.8 ± 2.6[a]	53.6 ± 5.5	50.5 ± 4.3	55.8 ± 5.7	53.8 ± 5.9	32.7 ± 3.5	37.2 ± 5.0	38.8 ± 3.2	42.6 ± 4.4	42.0 ± 2.3
CA1/DCX	18.8 ± 2.9	11.0 ± 1.5[c]	20.7 ± 3.9	35.2 ± 3.3	34.1 ± 6.7	26.7 ± 2.2	12.8 ± 4.3[e]	25.8 ± 3.2	27.7 ± 5.0	37.1 ± 3.8	37.3 ± 4.8
CA2/CA3/DCX	24.1 ± 6.1	6.42 ± 1.0[g]	17.7 ± 3.1	30.3 ± 3.7	19.7 ± 4.0	21.1 ± 4.2	10.4 ± 2.9[i]	26.3 ± 5.7	24.9 ± 5.1	42.5 ± 6.0	36.0 ± 6.1
Cortex/DCX	39.9 ± 1.6	26.0 ± 4.1[j]	32.7 ± 5.9	33.6 ± 2.6	52.1 ± 7.6	36.5 ± 6.3	23.3 ± 4.7[l]	40.8 ± 3.8	45.2 ± 3.3	49.4 ± 4.9	49.6 ± 3.1
DG/VEGF	40.1 ± 3.3	25.3 ± 2.3[b]	43.5 ± 2.1	46.0 ± 4.4	47.0 ± 5.4	46.0 ± 4.5	35.1 ± 3.6	28.9 ± 2.4	35.4 ± 2.7	42.6 ± 4.4	44.9 ± 4.3
CA1/VEGF	20.9 ± 4.1	15.2 ± 2.5[d]	27.6 ± 4.1	27.1 ± 2.8	37.4 ± 7.6	27.2 ± 3.3	15.7 ± 4.9[f]	27.2 ± 3.3	37.4 ± 4.8	37.1 ± 3.8	45.1 ± 4.7
CA2/CA3/VEGF	25.6 ± 7.9	8.8 ± 1.1[h]	34.1 ± 6.8	27.7 ± 4.8	25.5 ± 5.0	25.0 ± 2.9	15.6 ± 4.1	33.2 ± 6.2	40.5 ± 5.6	42.5 ± 6.0	44.9 ± 6.0
Cortex/VEGF	29.8 ± 4.7[m]	20.5 ± 2.4[k]	34.6 ± 5.1	28.0 ± 2.6	46.2 ± 7.6	25.9 ± 4.9	39.0 ± 4.1	30.7 ± 3.8	35.3 ± 6.2	49.4 ± 4.9	42.3 ± 2.8

Significant differences were observed:
In the DG region, E4-pretreated groups: DCX-stained cells, [a]vehicle vs. 10 mg/kg/day E4; VEGF-stained cells, [b]vehicle vs. 1, 5, 10, and 50 mg/kg/day E4
In the CA1 region, E4-pretreated groups: DCX-stained cells, [c]vehicle vs. 5 and 10 mg/kg/day E4; VEGF-stained cells, [d]vehicle vs. 10 mg/kg/day E4. E4-treated groups: DCX-stained cells, [e]vehicle vs. 10 and 50 mg/kg/day E4; VEGF-stained cells, [f]vehicle vs. 5, 10, and 50 mg/kg/day E4
In the CA2/CA3 region, E4-pretreated groups: DCX-stained cells, [g]vehicle vs. 5 mg/kg/day E4; VEGF-stained cells, [h]vehicle vs. 10 mg/kg/day E4. E4-treated groups: DCX-stained cells, [i]vehicle vs. 10 mg/kg/day
In the cortex, E4-pretreated groups: DCX-stained cells, [j]vehicle vs. 10 mg/kg/day E4; VEGF-stained cells, [k]vehicle vs. 10 mg/kg/day E4. E4-treated groups: DCX positively stained cells, [l]vehicle vs. 5, 10, and 50 mg/kg/day E4; VEGF-stained cells, [m]sham vs. 10 mg/kg/day E4
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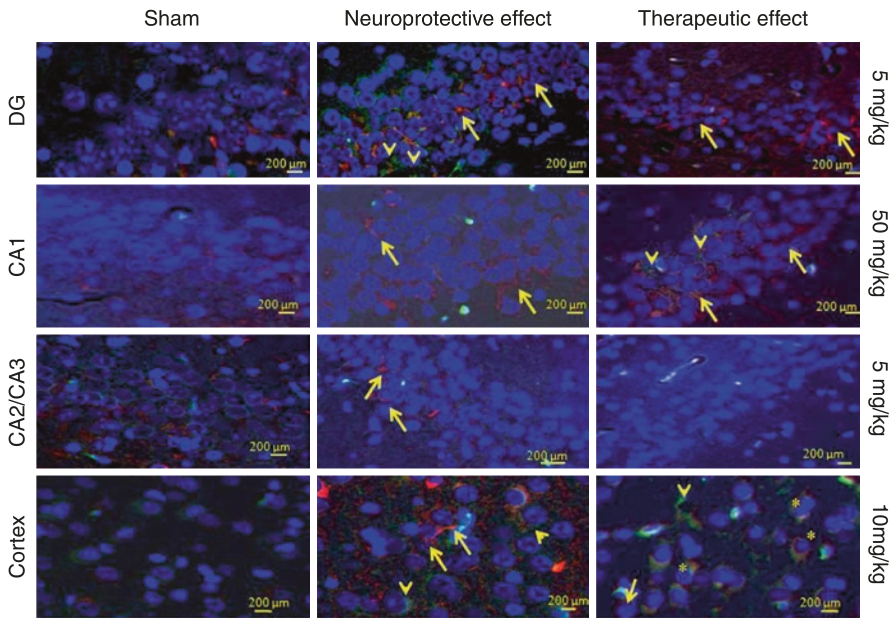

Fig. 4.6 Representative views of double-labeled immunofluorescence in the hippocampus and cortex of the rat pups pretreated or treated with E4. Double immunofluorescent staining was performed to determine the localization and expression of doublecortin (DCX) and vascular endothelial growth factor (VEGF) in different regions of hippocampus (dentate gyrus (DG), cornu ammonis 1 (CA1), cornu ammonis 2/3 (CA2/CA3), and cortex. Arrows denote DCX positively stained cells (red). Arrowheads denote VEGF positively stained cells (green). Asterisks indicate co-localization of DCX and VEGF positively stained cells. Scale bar: 200 μm. Reproduced from *Experimental Neurology* (Tskitishvili et al., 2014, 261:298–307)

respectively; in the CA2/CA3 region, neurogenesis was significantly upregulated in the 10 mg/kg/day E4 group alone, whereas in the cortex, upregulation of neuro- and cerebro-angiogenesis were more prominent in 5, 10, and 50 mg/kg/day and 10 mg/kg/day E4 groups, respectively (Table 4.2) [116] (Fig. 4.6).

Another issue was connected to the question whether pretreatment/treatment by E4 can affect the expression of brain damage markers in blood. Pretreatment by E4 in neuroprotective model resulted in significant downregulation of brain damage markers (S100B and GFAP) at a concentration of 50 mg/kg/day E4, whereas treatment by E4 after induction of experimental hypoxic-ischemic insult led to significant decrease of S100B and GFAP expression in all E4-treated groups (Table 4.3) [116].

4.4.2 Can We Use Estetrol in Combination with Other Steroids for Attenuation of HIE?

Dynamic changes in neurological field proposed new treatment strategies employing different compounds/steroids for attenuation of some neurological diseases.

Table 4.3 S100B and glial fibrillary acidic protein (GFAP) expression in blood serum of the rat pups pretreated/treated with E4

	E4 pretreatment		E4 treatment	
	S100B (pg/ml)	GFAP (pg/ml)	S100B (pg/ml)	GFAP (pg/ml)
Sham	344.6 ± 50.3[a]	407.0 ± 49.2	344.6 ± 50.3	407.6 ± 49.3
Vehicle	698.9 ± 57.3[b]	1003.9 ± 288.3[d]	1191.4 ± 211.2[e]	1762.3 ± 364.2[f]
1 mg/kg/day	560.2 ± 107.2	545.6 ± 85.4	665.7 ± 52.9	334.9 ± 23.9
5 mg/kg/day	395.0 ± 73.1	313.9 ± 36.8	628.3 ± 54.9	621.5 ± 90.7
10 mg/kg/day	715.7 ± 47.5[c]	630.4 ± 117.2	647.5 ± 41.3	479.1 ± 69.7
50 mg/kg/day	361.0 ± 32.9	300.4 ± 31.2	581.1 ± 73.5	460.0 ± 73.5

Significant differences were observed:
In E4-pretreated groups, S100B: [a]sham vs. vehicle and 10 mg/kg/day E4, [b]vehicle vs. 50 mg/kg/day E4, [c]10 mg/kg/day vs. 5 and 50 mg/kg/day E4. GFAP: [d]vehicle vs. sham and 50 mg/kg/day
In E4-treated groups, S100B: [e]vehicle vs. sham, 1, 5, 10, and 50 mg/kg/day E4. GFAP: [f]vehicle vs. sham, 1, 5, 10, and 50 mg/kg/day E4
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Like that several investigations already have demonstrated the neuroprotective efficacy of estradiol (E2) and progesterone (pregn-4-ene-3,20-dione) (P4) alone or in combination in different experimental models and clinical studies of neurological diseases: Parkinson's and Alzheimer's diseases, ischemic stroke, spinal cord injury, traumatic brain injury (TBI), and multiple sclerosis [12, 28, 29, 31, 32, 34, 37, 121–125]. P4-dependent neuroprotection is partly realized through attenuation of oxidative stress resulting from glutamate and glucose deprivation-induced toxicity [126–129]. Besides, in vitro, in primary hippocampal cell cultures, P4 might have protective effect against $FeSO_4$ and amyloid β-peptide-induced toxicity [130, 131]. Clinical studies in extremely preterm infants demonstrated reduction of the risk for cerebral palsy, spasticity, and ametropia at 5 years neurodevelopmental follow-up due to postnatal E2 and P4 combined replacement therapy [132]. Results of some studies employing combination of E2 and P4 are still controversial: though some studies have suggested that P4 does not affect the positive effects of E2 [128, 133, 134], others still argue that P4 might antagonize the positive effects of E2 [135–140].

As we know, the rat forebrain expresses high levels of progesterone receptors (PR) as early as E17–E18 in regions with important cognitive, motor, and visual functions; thus, the hippocampus has importance in establishment of early cortical circuitry. P4 neuroprotective effects are based on activation of inflammatory and oxidative mechanisms and the repair processes that usually follow the injury. As it was shown recently, the upregulation of nitric oxide synthase 2 (NOS-2), involved in production of nitric oxide free radicals, and pro-inflammatory IL-1β after ischemic events caused by MCAO is inhibited by progesterone treatment [141]. In adults after traumatic brain injury, P4 has an ability to reduce the proliferation of reactive astrocytes and inflammatory prostaglandin synthesis further leading to the reduction of edema and the blood-brain barrier leakage [122, 142]. P4 may induce a neuroprotective effect by upregulating expression of brain-derived neurotrophic factor (BDNF) or promoting increase of myelin basic protein expression (MBP)

[143], upregulating the inhibitory transmitter GABAa, and reducing the apoptosis by downregulation of NFκB [144–146]. Taken together, P4 along with E2 plays a critical role in neuronal developmental processes not only in prenatal period but in adulthood as well [147, 148].

Before starting a new stage of our studies with E4 and other steroids, it was important to define the working concentrations of P4 and E2. First, primary hippocampal neuronal cell cultures were treated after induction of the oxidative stress by P4 and E2 solely at doses starting from 1 nM until up to 1 mM. A significant downregulation of the LDH activity was observed at P4 concentration of 1 mM and E2 concentration of 100 nM (data not shown). Next we have performed treatment of primary hippocampal cell cultures after induction of oxidative stress with previously defined successful concentrations of E4 [116] which showed tremendous antioxidative and cell proliferative effects (650 μM, 3.25 mM, and 6.5 mM) alone or in combination with 1 mM PROG and/or E2 100 nM [117]. LDH activity as a marker of oxidative stress was significantly downregulated in all cultures exposed to steroids, especially in cultures exposed to different concentrations of E4 with E2 and P4 (Fig. 4.7a, b). Similar pattern of LDH activity was observed in cultures treated either by 6.5 mM E4 with E2 (Fig. 4.7a) or by different concentrations of E4 with P4 (Fig. 4.7b) compared to cultures treated by E4 alone [117]. The cell survival rate was significantly increased in cultures treated either by 6.5 mM E4 with/without E2 (Fig. 4.7c) or by 6.5 mM E4 with/without P4 (Fig. 4.7d) or by high doses of E4 with E2 and P4 (Fig. 4.7c, d). Furthermore, cells exposed to 6.5 mM E4 with/without E2 had significantly higher cell survival rate than the cultures treated by 650 μM E4 with/without E2 (Fig. 4.7c), though the dose-dependent pattern was more prominent when different concentrations of E4 were used with/without P4 (Fig. 4.7d). Cells treated by 6.5 mM E4 and P4 or treated by 6.5 mM E4 with E2 and P4 had significantly higher survival rate than the cells treated only by E4 (Fig. 4.7d) or those cells treated by 6.5 mM E4 with/without E2 (Fig. 4.7c) and by the lower doses of E4 combined with E2 and P4, respectively (Fig. 4.7c, d) [117].

Interesting observations were monitored in vivo. In neuroprotective model, when steroids were used before induction of brain injury, rectal temperature immediately after hypoxic-ischemic (HI) insult (at 0 h time point) was significantly increased only in animals from the vehicle group, whereas 2 h later the rectal temperature was significantly decreased in groups pretreated by combination of 5 mg/kg/day E4 and 1.6 mg/kg/day P4 with/without 136 ng/kg/day E2 (Fig. 4.8a) [117]. In therapeutic model, 2 h after HI insult, groups treated by combination of any dose of E4 with 16 mg/kg/day P4 plus 136 ng/kg/day E2 had significantly decreased rectal temperature than the vehicle group or the groups treated by the same doses of E4 with 1.6 mg/kg/day P4 and E2. Combination of 10 mg/kg/day E4 with 16 mg/kg/day P4 with or without 136 ng/kg/day E2 also 10 mg/kg/day E4 alone or combined with 1.6 mg/kg/day P4 significantly downregulated the rectal temperature (Fig. 4.8b) [117]. At 4 h

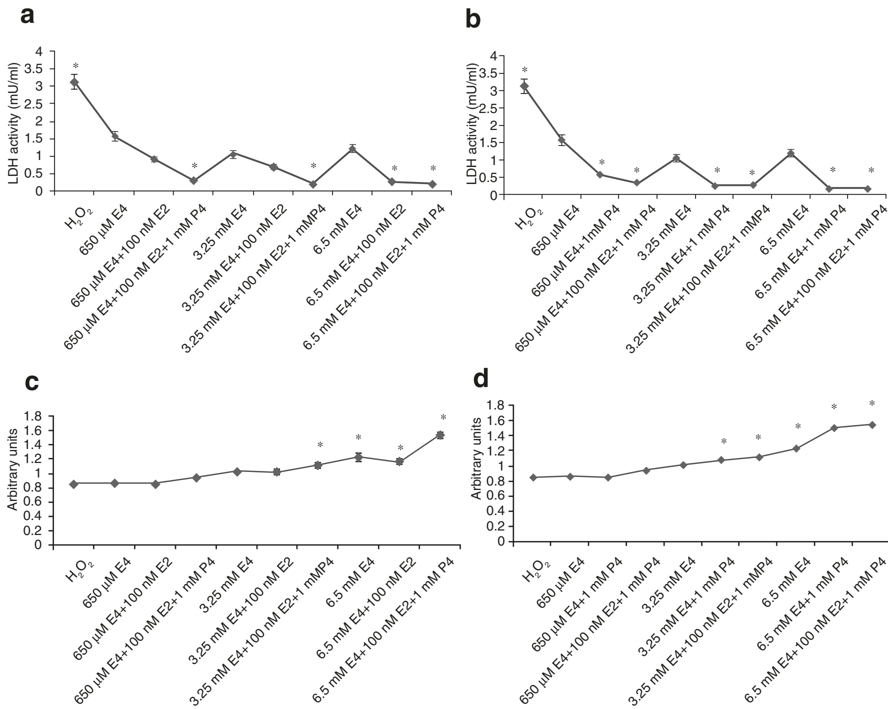

Fig. 4.7 Effect of E4 alone or in combination with P4 and/or E2 on LDH activity and cell viability in primary hippocampal cell cultures subjected to the H_2O_2-induced oxidative stress. (**a**–**d**) Primary hippocampal neuronal cells were treated with 650 μM, 3.25 mM, and 6.5 mM of estetrol alone or in combination with 100 nM E2 and/or 1 mM P4 for 1 h after induction of oxidative stress by 100 μM of H_2O_2 for 30 min. (**a**, **b**). LDH activity was significantly downregulated in all the study groups compared to the H_2O_2-treated group. The LDH activity level was significantly lower in cultures treated either with 6.5 mM E4 or 100 nM E2 (**a**) or in cultures treated by any dose of E4 along with 1 mM P4 than in cultures treated by E4 alone (**b**) as well as in cultures combinedly treated by any dose of E4 along with 1 mM P4 and 100 nM E2 than in cultures treated by E4 alone (**a**, **b**). (**c**) Cell survival was significantly upregulated in cultures treated by 6.5 mM E4 with/without 1 mM P4 or by 3.25 mM and 6.5 mM E4 with 100 nM E2 and 1 mM P4 in comparison with H_2O_2-treated cultures. Cells exposed to 6.5 mM E4 with/without 100 nM E2 had significantly higher cell survival rate than the cultures treated by 650 μM E4 with/without 100 nM E2. Cells combinedly treated by 6.5 mM E4 with 100 nM E2 and 1 mM P4 had significantly higher survival level than the cells treated by 6.5 mM E4 with/without 100 nM E2. (**d**) Cell survival was significantly upregulated in cultures treated by 6.5 mM E4 with/without 1 mM P4 or by 3.25 mM and 6.5 mM E4 with 100 nM E2 and 1 mM P4 than in H_2O_2-treated cultures. The dose-dependent pattern was observed when 650 μM, 3.25 mM, and 6.5 mM E4 were used with/without 1 mM P4. (**c**, **d**) Cell cultures combinedly treated by 6.5 mM E4 with 100 nM E2 and 1 mM P4 had significantly higher survival level than the cells treated by 650 μM and 3.25 mM E4 in combination with 100 nM E2 and 1 mM P4. All measurements are expressed as mean ± SEM. $^*p \leq 0.05$. Reproduced from *Oncotarget* (Tskitishvili et al., 2016, 7(23):33722–43)

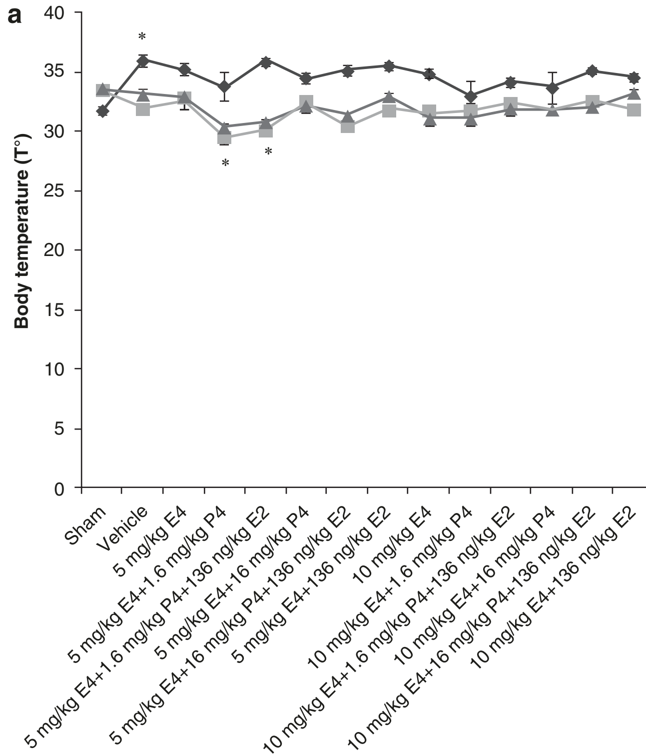

Fig. 4.8 Postoperative rectal temperature and body weight of rat pups. (**a**). In neuroprotective model, immediately after hypoxic-ischemic (HI) insult (at 0 h), the rectal temperature was significantly increased only in the vehicle group than in the sham group, whereas 2 h later the rectal temperature was significantly decreased in pretreated by 5 mg/kg/day E4 and 1.6 mg/kg/day P4 with/without 136 ng/kg/day E2 groups compared to the sham group. Four hours later no significant difference was observed among the study groups. (**b**) In therapeutic model, between the study groups immediately after HI insult, no significant differences were detected, whereas 2 h later groups treated by combination of 5 mg/kg/day or 10 mg/kg/day E4 with 16 mg/kg/day P4 plus 136 ng/kg/day E2 had significantly decreased rectal temperature than the vehicle group or the groups treated by the same doses of E4 with 1.6 mg/kg/day P4 and E2. Moreover, combination of 10 mg/kg/day E4 with 16 mg/kg/day P4 and 136 ng/kg/day E2 significantly downregulated the rectal temperature compared to the sham group or the group treated by 10 mg/kg/day E4 and 16 mg/kg/day P4 (Fig. 4.2b). Also, the groups treated by 10 mg/kg/day E4 alone or combined with 1.6 mg/kg/day P4 had significantly decreased rectal temperature compared to the group treated by 10 mg/kg/day E4 with 1.6 mg/kg/day P4 and 136 ng/kg/day E2. At 4 h after HI event, animals treated by 5 mg/kg/day or 10 mg/kg/day E4 and 136 ng/kg/day E2 with/without 16 mg/kg/day P4 had significantly decreased rectal temperature along with the sham group compared to animals treated by single doses of E4 (Fig. 4.2b). The same pattern was observed between groups treated by 10 mg/kg/day E4 with 1.6 mg/kg/day P4 and 136 ng/kg/day E2 and the group treated by E4 alone. Treatment by 5 mg/kg/day E4 with 16 mg/kg/day P4 and 136 ng/kg/day E2 significantly decreased the rectal temperature than the treatment by the same combination of compounds with 1.6 mg/kg/day P4 (Fig. 4.2b). All measurements are expressed as mean ± SEM. $^{*}p \leq 0.05$. Reproduced from *Oncotarget* (Tskitishvili et al., 2016, 7(23):33722–43)

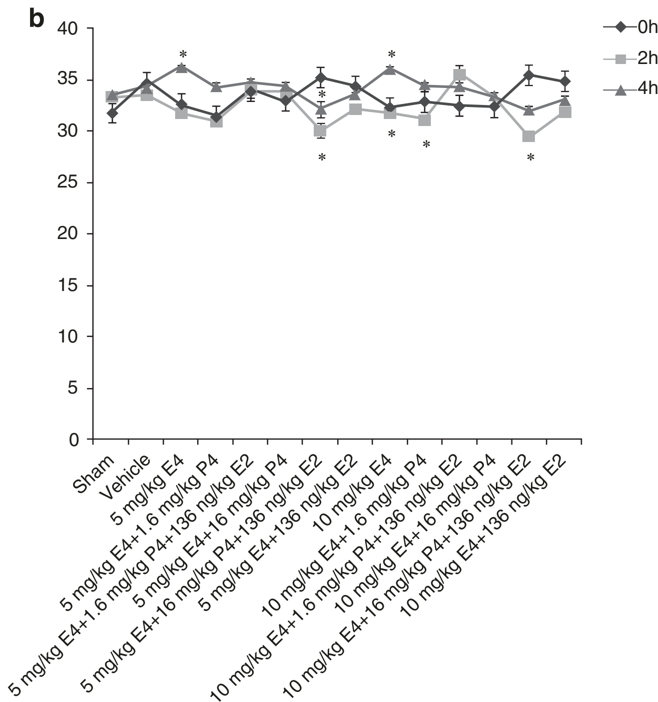

Fig. 4.8 (continued)

after HI insult, animals treated by E4 and E2 with/without 1.6 mg/kg/day/16 mg/kg/day P4 had significantly decreased rectal temperature compared to animals treated by single doses of E4 (Fig. 4.8b). Treatment by 5 mg/kg/day E4 with 16 mg/kg/day P4 and E2 significantly decreased the rectal temperature than the treatment by the same combination of compounds with 1.6 mg/kg/day P4 (Fig. 4.8b) [117].

As it is shown in Table 4.4, animals pretreated by a combination of 5 mg/kg/day E4 and 16 mg/kg/day P4 before experimental brain injury had significantly higher body weight than animals from the vehicle, sham, and combinedly pretreated by 5 mg/kg/day E4 and E2 groups, and the brain-body weight ratio was significantly higher in groups pretreated by 5 mg/kg/day E4 and 136 ng/kg/day E2 in combination either with 1.6 or 16 mg/kg/day P4. In groups treated by steroids after experimental brain damage, only animals from 10 mg/kg/day E4 group had significantly higher brain weight compared to the vehicles (Table 4.4) without affecting the brain-body weight ratio [117].

Histochemical studies of coronal sections from rat pups' brains pretreated/treated by the vehicle showed obvious injury of the hippocampus at the left carotid artery occlusion (damaged) side which was extended to the cortex at the same side (Figs. 4.9 and 4.10A(b), B(b)). It was also interesting to observe the damage of the cortex of the left hemisphere in animals that were pretreated/treated by combination of E4 and E2 (Fig. 4.9) [117].

Table 4.4 Body and brain weights of rat pups from study groups

Groups	Body weight (g)		Brain weight (g)	*p*
	P7	P14		
Pretreatment				
Sham	12.04 ± 0.52	26.96 ± 0.73	1.20 ± 0.01	
Vehicle	12.52 ± 0.41	27.86 ± 0.65	1.15 ± 0.01	
5 mg/kg E4	15.03 ± 0.68	24.61 ± 1.19	1.18 ± 0.03	
5 mg/kg E4 + 1.6 mg/kg P4	12.89 ± 0.46	26.63 ± 1.30	1.21 ± 0.03	
5 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	11.93 ± 0.36	22.04 ± 0.66	1.19 ± 0.03	
5 mg/kg E4 + 16 mg/kg P4	15.94 ± 0.29	25.58 ± 1.47	1.17 ± 0.02	[a]
5 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	14.27 ± 0.70	21.55 ± 0.68	1.16 ± 0.03	
5 mg/kg E4 + 136 ng/kg E2	11.94 ± 0.59	23.91 ± 1.34	1.12 ± 0.03	
10 mg/kg E4	13.35 ± 0.47	27.10 ± 0.83	1.19 ± 0.02	
10 mg/kg E4 + 1.6 mg/kg P4	12.83 ± 0.66	25.57 ± 0.99	1.25 ± 0.01	
10 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	13.59 ± 0.50	27.91 ± 1.18	1.23 ± 0.02	
10 mg/kg E4 + 16 mg/kg P4	13.55 ± 0.34	26.46 ± 1.20	1.23 ± 0.03	
10 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	13.15 ± 0.25	27.99 ± 1.03	1.24 ± 0.01	
10 mg/kg E4 + 136 ng/kg E2	12.47 ± 0.52	25.69 ± 0.93	1.21 ± 0.03	
Treatment				
Sham	12.04 ± 0.52	26.96 ± 0.73	1.20 ± 0.01	
Vehicle	13.43 ± 0.35	26.63 ± 0.71	1.17 ± 0.02	
5 mg/kg E4	14.14 ± 0.62	28.21 ± 1.23	1.28 ± 0.01	
5 mg/kg E4 + 1.6 mg/kg P4	14.08 ± 0.65	27.16 ± 0.66	1.23 ± 0.02	
5 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	13.66 ± 0.43	25.67 ± 0.88	1.29 ± 0.01	
5 mg/kg E4 + 16 mg/kg P4	13.51 ± 0.41	27.11 ± 1.18	1.26 ± 0.03	
5 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	15.03 ± 0.49	28.13 ± 1.37	1.22 ± 0.03	
5 mg/kg E4 + 136 ng/kg E2	13.93 ± 0.37	24.43 ± 2.07	1.15 ± 0.05	
10 mg/kg E4	14.25 ± 0.59	30.82 ± 0.54	1.34 ± 0.01	[b]
10 mg/kg E4 + 1.6 mg/kg P4	13.83 ± 0.66	24.97 ± 0.89	1.20 ± 0.02	
10 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	13.44 ± 0.47	25.48 ± 1.22	1.25 ± 0.02	
10 mg/kg E4 + 16 mg/kg P4	13.93 ± 0.39	25.98 ± 0.74	1.32 ± 0.01	
10 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	14.17 ± 0.51	32.41 ± 0.96	1.26 ± 0.02	
10 mg/kg E4 + 136 ng/kg E2	15.04 ± 0.42	28.64 ± 2.76	1.22 ± 0.04	

Significant differences were observed: [a]body weight at P7, 5 mg/kg/day E4 + 16 mg/kg/day P4 group vs. sham, vehicle, and 5 mg/kg/day E4 + 136 ng/kg/day E2 in pretreated groups; [b]brain weight, 10 mg/kg/day E4 vs. vehicle in treated groups

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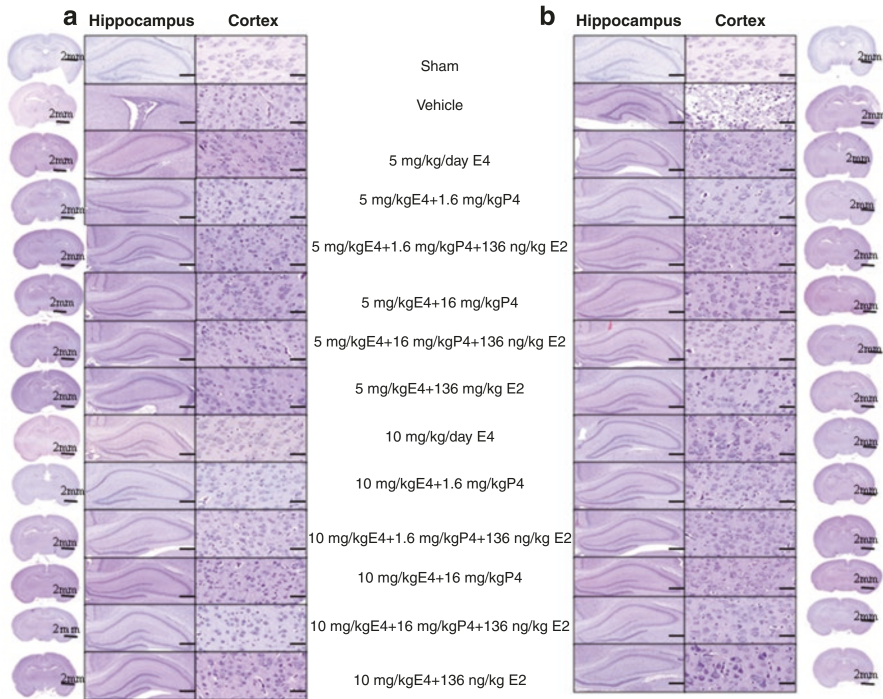

Fig. 4.9 Representative views of hematoxylin-eosin-stained brain coronal sections from rat pups pretreated/treated by E4 alone or in combination with P4 and/or E2. Paraffin-embedded brain samples were sliced into 5-μm-thick coronal sections at the hippocampus level. Sections were deparaffinized and rehydrated, and hematoxylin and eosin staining was performed. Brain coronal sections (scale bar, 2 mm) with hippocampus region (scale bar, 500 μm) and the cortex (scale bar, 100 μm) from pretreated (**a**) and treated (**b**) study groups are presented. Reproduced from *Oncotarget* (Tskitishvili et al., 2016, 7(23):33722–43)

Cell counting per visual field in pretreated groups showed (Table 4.5) that in the DG region there were significant differences between the study groups as follows: sham and the vehicle groups or groups pretreated by 5 mg/kg/day E4 and E2 with/without 16 mg/kg/day P4 (Fig. 4.9a and 4.10A(c), (d), respectively) or by 10 mg/kg/day E4 with E2 and 16 mg/kg/day P4 (Fig. 4.9a). In the same region of the hippocampus, significantly higher number of intact cells was observed in animals pretreated by 5 mg/kg/day E4 (Fig. 4.10A(c)) and 10 mg/kg/day E4 (Fig. 4.10A(f)) alone or in combination with 1.6 mg/kg/day P4 and/or E2, also in animals pretreated with 10 mg/kg/day E4 and 16 mg/kg/day P4 (Table 4.5), as well as between animals pretreated by 10 mg/kg/day E4 alone or in combination with E2 and/or any

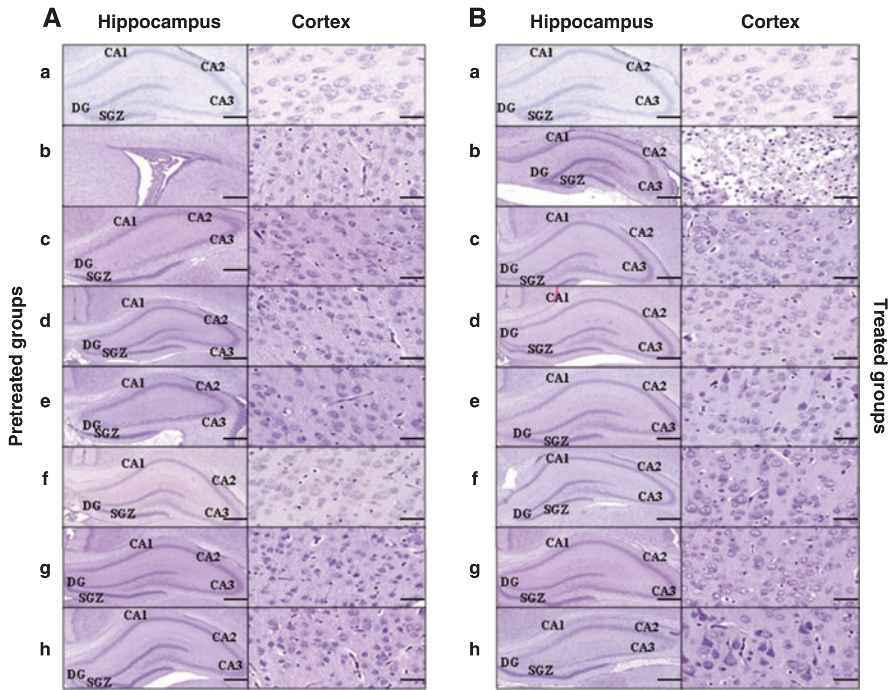

Fig. 4.10 Hematoxylin-eosin staining of the brain coronal sections from rat pups pretreated/treated by E4 alone or in combination with P4 and/or E2. Brain coronal sections (scale bar, 2 mm) with hippocampus region (scale bar, 500 μm) and cortex (scale bar, 100 μm) from pretreated **A** and treated **B** study groups are shown: sham (a), vehicle (b), 5 mg/kg/dayE4 (c), 5 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2 (d), 5 mg/kg/day E4 + 136 ng/kg/day E2 (e), 10 mg/kg/day E4 (f), 10 mg/kg/day E4 + 16 mg/kg/day P4 (g), 10 mg/kg/day E4 + 136 ng/kg/day E2 (h). Reproduced from *Oncotarget* (Tskitishvili et al., Use of estetrol with other steroids for attenuation of neonatal hypoxic-ischemic brain injury: to combine or not to combine? *Oncotarget*. 2016, 7(23):33722–43)

concentration of P4, and the sham group (Table 4.5) [117]. In the SGZ the sham group had significantly higher intact cell counting than animals pretreated by 5 mg/kg/day E4 with E2 (Figs. 4.9a and 4.10A(h)), whereas the number of intact cells was significantly upregulated in the groups pretreated by different doses of E4 alone or combined with 16 mg/kg/day P4. The same pattern of significant difference was observed in animals pretreated by 5 mg/kg/day E4 in combination with 16 mg/kg/day P4 plus E2 and the vehicle group (Table 4.5) [117].

In treated groups (Figs. 4.9b and 4.10B), in the DG region, the number of intact cells was significantly different between the groups: vehicle and sham, also groups treated by combination of different doses of E4 either with any dose of P4 or E2. Intact cell number was significantly downregulated in animals combinedly treated by 10 mg/kg/day E4 and E2 (Fig. 4.10B(h)) compared to the sham group (Table 4.5). In the SGZ region, significant differences were observed between the vehicle group and the animals treated by different doses of E4 with 1.6 mg/kg/day P4 or E2, also

Table 4.5 Intact cell counting/per visual field in the hippocampus and cortex in hematoxylin-eosin-stained sections

Groups	DG	SGZ	CA1	CA2/CA3	Cortex
Pretreatment					
Sham	202.70 ± 18.28[a]	89.80 ± 8.57[f]	55.40 ± 3.34	44.70 ± 2.38	47.20 ± 3.90
Vehicle	76.30 ± 10.23[b]	33.20 ± 5.22[g]	48.30 ± 6.65	33.80 ± 3.93	33.30 ± 3.24
5 mg/kg E4	183.40 ± 11.96	72.10 ± 5.93	48.60 ± 2.57	51.80 ± 3.56	39.80 ± 2.74
5 mg/kg E4 + 1.6 mg/kg P4	168.80 ± 17.52	62.50 ± 6.97	54.00 ± 3.11	45.30 ± 2.89	36.50 ± 3.19
5 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	168.80 ± 4.71	67.7 ± 3.32	53.60 ± 3.47	40.80 ± 3.06	43.60 ± 4.02
5 mg/kg E4 + 16 mg/kg P4	148.60 ± 12.97	68.60 ± 5.15	51.70 ± 5.69	54.60 ± 7.00	41.90 ± 4.51
5 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	167.40 ± 13.96	73.90 ± 7.96	45.00 ± 6.07	44.40 ± 6.59	40.20 ± 3.60
5 mg/kg E4 + 136 ng/kg E2	111.40 ± 6.69	42.90 ± 3.09	56.20 ± 3.76	38.50 ± 2.85	36.50 ± 3.37
10 mg/kg E4	227.30 ± 14.18[c]	86.10 ± 5.84	64.90 ± 3.63	42.40 ± 2.49	39.60 ± 2.86
10 mg/kg E4 + 1.6 mg/kg P4	166.90 ± 11.97	64.30 ± 5.26	58.30 ± 3.53	45.30 ± 2.92	45.40 ± 5.70
10 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	123.4 ± 7.25	54.20 ± 4.84	65.90 ± 4.91	46.10 ± 7.72	41.90 ± 2.65
10 mg/kg E4 + 16 mg/kg P4	174.60 ± 12.38	73.50 ± 6.97	58.40 ± 4.74	46.30 ± 4.57	38.40 ± 5.45
10 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	115.70 ± 6.51	52.70 ± 2.49	45.00 ± 3.63	46.30 ± 5.02	36.90 ± 3.485
10 mg/kg E4 + 136 ng/kg E2	131.70 ± 8.94	56.30 ± 3.02	57.70 ± 2.46	43.30 ± 1.89	39.50 ± 4.37
Treatment					
Sham	202.70 ± 18.28	89.80 ± 8.57[h]	55.40 ± 3.34	44.70 ± 32.38[i]	47.20 ± 3.90
Vehicle	74.00 ± 10.61[d]	39.10 ± 6.79	41.80 ± 6.86	24.30 ± 4.15	20.30 ± 2.33[j]
5 mg/kg E4	142.90 ± 5.63	60.40 ± 3.55	56.30 ± 3.45	32.90 ± 31.62	32.70 ± 2.64
5 mg/kg E4 + 1.6 mg/kg P4	138.80 ± 14.01	45.20 ± 4.20	53.20 ± 5.67	34.60 ± 33.21	42.30 ± 3.72
5 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	160.00 ± 7.29	61.10 ± 3.94	56.50 ± 2.79	39.50 ± 31.46	41.40 ± 4.79
5 mg/kg E4 + 16 mg/kg P4	175.50 ± 7.84	61.30 ± 3.08	55.10 ± 3.85	42.5 ± 31.87	50.90 ± 4.87
5 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	164.00 ± 12.45	58.4 ± 4.92	57.50 ± 4.17	35.6 ± 33.45	37.00 ± 4.17
5 mg/kg E4 + 136 ng/kg E2	128.50 ± 12.15	54.10 ± 4.37	54.20 ± 4.95	38.00 ± 34.13	46.00 ± 4.51
10 mg/kg E4	150.20 ± 9.43	57.30 ± 5.19	51.20 ± 2.82	33.50 ± 0.98	46.60 ± 1.83
10 mg/kg E4 + 1.6 mg/kg P4	132.20 ± 13.68	41.90 ± 2.88	47.10 ± 6.64	37.60 ± 33.36	31.30 ± 3.83

(continued)

Table 4.5 (continued)

Groups	DG	SGZ	CA1	CA2/CA3	Cortex
10 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	156.00 ± 6.11	56.70 ± 2.45	61.50 ± 2.88	39.80 ± 31.30	38.80 ± 4.81
10 mg/kg E4 + 16 mg/kg P4	168.40 ± 4.38	65.40 ± 3.09	57.50 ± 1.73	40.40 ± 31.83	48.10 ± 4.02
10 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	150.00 ± 9.92	54.40 ± 4.01	50.90 ± 4.59	32.60 ± 32.82	39.10 ± 3.30
10 mg/kg E4 + 136 ng/kg E2	103.40 ± 13.47[e]	41.50 ± 5.55	48.40 ± 6.86	28.90 ± 35.34	39.10 ± 3.45

Significant differences were observed:

In the DG region

Pretreated groups: [a]sham vs. 5 mg/kg/day E4 + 136 ng/kg/day E2, 5 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day + 16 mg/kg/day P4 + 136 ng/kg/day E2; [b]vehicle vs. sham, 5 mg/kg/day E4, 5 mg/kg/day E4 + 1.6 mg/kg/day P4, 5 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4, 10 mg/kg/day E4 + 1.6 mg/kg/day P4, 10 mg/kg/day E4 + 16 mg/kg/day P4; [c]10 mg/kg/day E4 vs. vehicle, sham, 10 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 136 ng/kg/day E2

Treated groups: [d]vehicle vs. sham, 5 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 5 mg/kg/day E4 + 16 mg/kg/day P4, 5 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4, 10 mg/kg/day E4 + 16 mg/kg/day P4, 10 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2; [e]10 mg/kg/day E4 + 136 ng/kg/day E2 vs. sham group in treated groups

In the SGZ

Pretreated groups: [f]sham vs. 5 mg/kg/day E4 + 136 ng/kg/day E2; [g]vehicle vs. sham, 5 mg/kg/day E4, 5 mg/kg/day + 16 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4, 10 mg/kg/day E4 + 16 mg/kg/day P4

Treated groups: [h]sham vs. vehicle, 5 mg/kg/day + 136 ng/kg/day E2, 5 mg/kg/day + 1.6 mg/kg/day P4, 10 mg/kg/day E4 + 1.6 mg/kg/day P4, 10 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 136 ng/kg/day E2

In the CA2/CA3 region

Treated groups: [i]sham vs. vehicle in treated groups

In the cortex

Treated groups: [j]vehicle vs. sham, 5 mg/kg/day + 16 mg/kg/day P4, 10 mg/kg/day, 10 mg/kg/day + 16 mg/kg/day P4

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the animals treated by 10 mg/kg/day E4 with E2 and different doses of P4 (Fig. 4.9b and Table 4.5). In the CA2/CA3 region, significant differences were observed only among animals from sham and the vehicle groups, whereas in the cortex, significantly higher number of intact cells was detected except for the sham group in groups treated either by different doses of E4 in combination with 16 mg/kg/day P4 or 10 mg/kg/day E4 alone (Fig. 4.10B) and the vehicle group (Table 4.5) [117].

By using the MAP staining, we have evaluated the gray matter loss in study groups. MAP-2 negatively stained areas corresponded to the damaged areas in the left hemisphere (the hippocampus and the cortex) (Fig. 4.9a, b) [117]. MAP-2 positively stained area ratio was significantly upregulated in animals pretreated by 10 mg/kg/day E4 alone along with animals from sham group (Figs. 4.11a and 4.12a), whereas after treatment with different combinations of steroids, MAP-2 positive area ratio was significantly higher in groups treated by E4 alone or in combination with 16 mg/kg/day P4 compared to the vehicle group (Figs. 4.11b and 4.12b). The similar pattern showed animals combinedly treated by 5 mg/kg/day E4 with 1.6 mg/kg/day P4 and E2. Treatment with 5 mg/kg/day E4 alone or either with 1.6 mg/kg/P4 and E2 or 16 mg/kg/day P4 restored the MAP-2 positive area ratio almost to the sham level (Figs. 4.11b and 4.12b) [117].

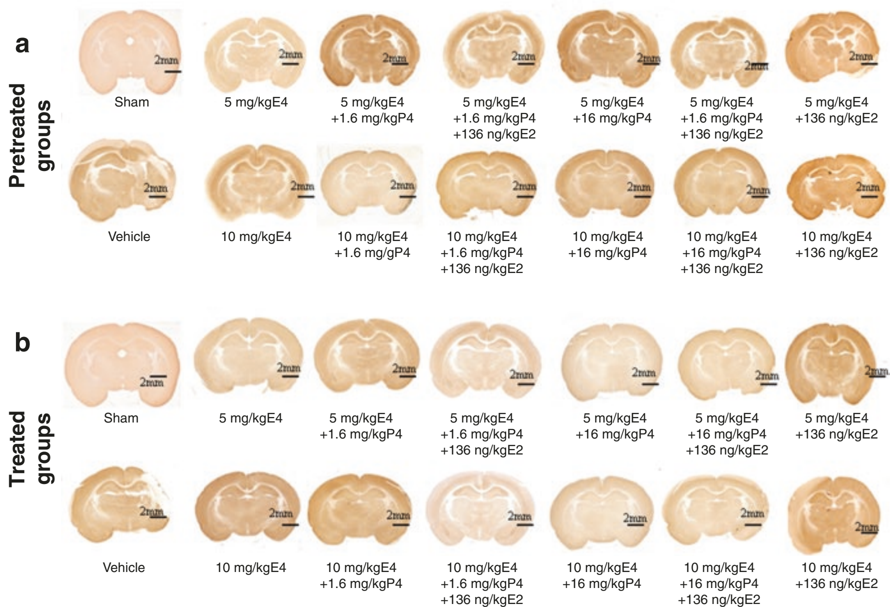

Fig. 4.11 Representative views of MAP-2-stained coronal brain sections from groups pretreated or treated with E4 alone or in combination with P4 and/or E2. From left to right are presented MAP-2-stained sections from pretreated (**a**) and treated (**b**) groups. In sections from the vehicle-pretreated/treated animals was observed an existence of MAP-2 negatively stained areas in the hippocampus and the cortex at the left, damaged side. Scale bar: 2 mm. Reproduced from *Oncotarget* (Tskitishvili et al., 2016, 7(23):33722–43)

We have also studied the possible effect of combined use of E4 with other steroids on neuro- and cerebro-angiogenesis by using specific markers DCX and VEGF, respectively, as previously (Fig. 4.13) [117]. In groups pretreated by different combinations of steroids before experimental HI insult in the DG region, neurogenesis and angiogenesis were significantly upregulated in 5 mg/kg/day E4 and 10 mg/kg/day E4 along with 1.6 mg/kg/day P4 and E2, respectively (Table 4.6); in the CA1 region, significant differences in DCX expression were observed between the sham and the vehicle groups, though VEGF expression was significantly increased in animals combinedly pretreated by 5 mg/kg/day E4 and 16 mg/kg/day P4 (Table 4.6); in the CA2/CA3 region, expressions of DCX and VEGF were significantly different only between the sham and the vehicle groups, whereas in the cortex between the sham and 5 mg/kg/day E4 plus 16 mg/kg/day P4 groups (Table 4.6) [117]. Notably, pretreatment by combination of E4 and E2 did not show any positive result for neuro- and angiogenesis (Fig. 4.13) [117].

Treatment of animals after HI insult with different combinations of steroids resulted in significant upregulation of neurogenesis in the hippocampus in animals

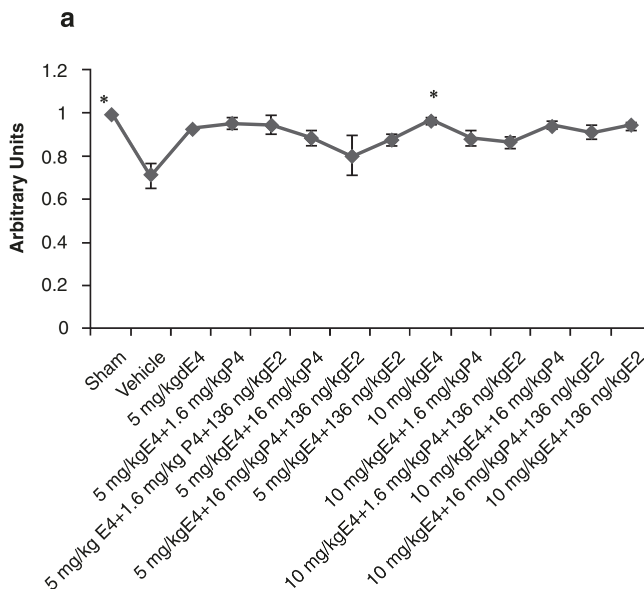

Fig. 4.12 MAP-2 staining of brain coronal sections from rat pups pretreated/treated by E4 alone or in combination with P4 and/or E2. For evaluation of gray matter loss, MAP-2 staining was performed. (**a**) Among pretreated groups the MAP-2 positively stained area ratio was significantly upregulated in animals pretreated by 10 mg/kg/day E4 alone than in the vehicles as well as in animals from sham group. (**b**) After treatment with different combinations of steroids, MAP-2-positive area ratio was significantly higher along with the sham group in groups treated by 5 or 10 mg/kg/day E4 alone or in combination with 16 mg/kg/day P4 compared to the vehicle group. The similar pattern was observed in animals combinedly treated by 5 mg/kg/day E4 with 1.6 mg/kg/day P4 and 136 ng/kg/day E2. Ten samples from each group were analyzed. The ratio of the MAP-2-positive area in sham-operated animals was considered as 1.0 by default. All measurements are expressed as mean ± SEM. *$p \leq 0.05$. Reproduced from *Oncotarget* (Tskitishvili et al., 2016, 7(23):33722–43)

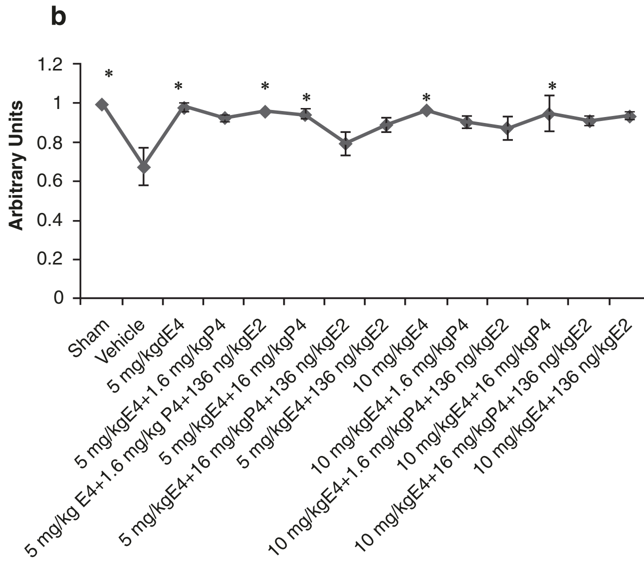

Fig. 4.12 (continued)

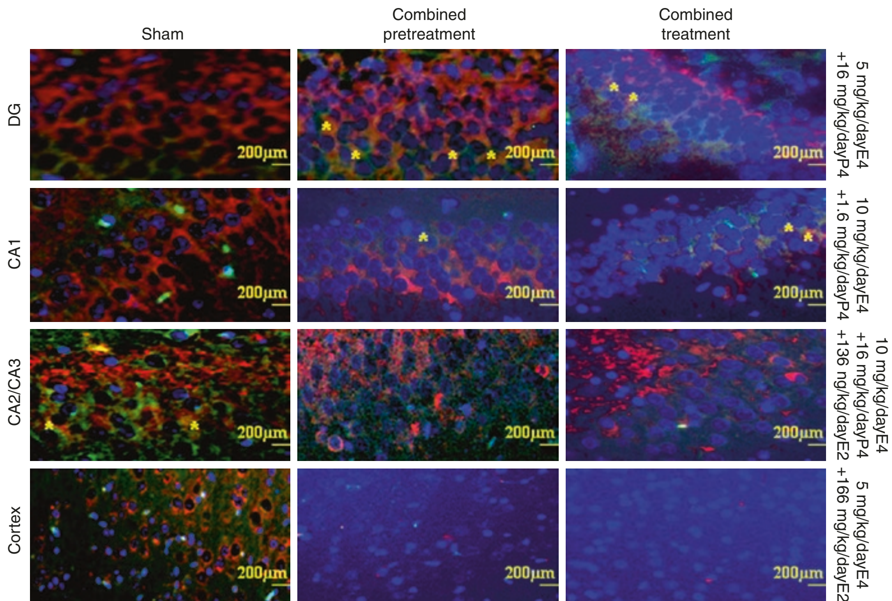

Fig. 4.13 Representative views of double-labeled immunofluorescent sections from different regions of the hippocampus and the cortex from groups pretreated/treated with E4 alone or in combination with P4 and/or E2. To determine the localization and expression of DCX and VEGF in different regions of the hippocampus and the cortex, the double immunofluorescent staining was performed. Red cells denote the DCX positively stained cells, whereas green cells denote the VEGF positively stained cells. Asterisks indicate co-localization of DCX and VEGF positively stained cells. Scale bar: 200 µm. Reproduced from *Oncotarget* (Tskitishvili et al., 2016, 7(23):33722–43)

Table 4.6 Percentage of the DCX and VEGF positively stained cells in combinedly pretreated/treated groups

	DG	CA1	CA2/CA3	Cortex	DG	CA1	CA2/CA3	Cortex
Groups	DCX	DCX	DCX	DCX	VEGF	VEGF	VEGF	VEGF
Pretreatment								
Sham	59.73 ± 3.44	52.34 ± 30.99	51.25 ± 2.42	63.34 ± 2.17	52.38 ± 1.58	51.87 ± 3.68	55.79 ± 2.92	58.29 ± 2.98
Vehicle	30.90 ± 3.97[a]	16.78 ± 32.36[f]	15.97 ± 2.35[j]	29.48 ± 1.98[o]	31.25 ± 3.70[b]	20.76 ± 2.71[g]	20.05 ± 3.01[k]	28.47 ± 2.13[p]
5 mg/kg E4	60.29 ± 5.66	45.97 ± 34.40	32.38 ± 5.03[m]	51.47 ± 3.98	42.81 ± 2.68	49.99 ± 3.94	43.80 ± 3.68	46.00 ± 2.55
5 mg/kg E4 + 1.6 mg/kg P4	45.16 ± 3.26	42.67 ± 35.07	34.03 ± 5.62	51.89 ± 5.26	38.60 ± 3.32	44.96 ± 5.04	42.96 ± 3.70	40.31 ± 4.95
5 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	58.55 ± 5.37	37.08 ± 32.67	22.68 ± 2.81	53.98 ± 3.81	47.71 ± 4.36	36.17 ± 3.17	31.14 ± 2.87	39.23 ± 2.69
5 mg/kg E4 + 16 mg/kg P4	56.23 ± 4.02	51.34 ± 34.00	49.02 ± 9.09	71.46 ± 4.89	44.53 ± 2.50	59.56 ± 3.26	51.57 ± 6.77	60.22 ± 4.09
5 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	53.40 ± 3.56	32.41 ± 39.50	27.43 ± 9.36	53.06 ± 3.10	46.67 ± 2.59	42.49 ± 8.47	41.07 ± 7.66	49.67 ± 4.23
5 mg/kg E4 + 136 ng/kg E2	33.49 ± 4.53	42.61 ± 35.66	36.19 ± 3.25	52.05 ± 3.92	46.84 ± 2.56	50.07 ± 4.74	46.74 ± 3.87	43.20 ± 4.02
10 mg/kg E4	50.43 ± 4.56	39.96 ± 33.60	26.58 ± 2.8	56.12 ± 6.10	51.44 ± 3.29	49.21 ± 3.49	38.31 ± 4.33	52.90 ± 5.81
10 mg/kg E4 + 1.6 mg/kg P4	45.02 ± 5.92	31.59 ± 36.83	18.11 ± 3.46	44.88 ± 7.95	42.77 ± 3.97	39.57 ± 6.49	39.64 ± 6.07	46.93 ± 6.38
10 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	53.25 ± 3.17	38.75 ± 35.03	36.12 ± 4.79	56.97 ± 7.53	55.34 ± 2.35	34.83 ± 3.38	42.80 ± 5.43	52.93 ± 5.54
10 mg/kg E4 + 16 mg/kg P4	44.50 ± 2.96	32.18 ± 35.74	27.73 ± 6.12	49.22 ± 4.72	47.05 ± 3.30	32.57 ± 4.74	34.51 ± 7.05	38.10 ± 4.57
10 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	45.86 ± 3.28	30.73 ± 34.99	22.41 ± 4.47	53.04 ± 5.04	43.87 ± 3.22	37.30 ± 3.13	36.25 ± 6.02	38.03 ± 3.25
10 mg/kg E4 + 136 ng/kg E2	39.25 ± 1.63	41.02 ± 34.96	46.71 ± 3.76	52.02 ± 3.34	48.40 ± 2.66	49.99 ± 3.20	51.74 ± 5.20	45.40 ± 3.63
Treatment								
Sham	59.73 ± 3.44[d]	52.43 ± 0.99	51.25 ± 2.42	63.34 ± 2.17	52.38 ± 1.58	51.87 ± 3.68	55.79 ± 2.92	58.29 ± 2.98
Vehicle	30.25 ± 4.90[c]	23.17 ± 4.43[h]	16.96 ± 3.67[l]	31.01 ± 3.90[q]	29.87 ± 2.88[e]	21.95 ± 2.48[i]	22.93 ± 2.37[n]	27.85 ± 2.80[r]
5 mg/kg E4	55.72 ± 2.91	36.63 ± 4.00	15.09 ± 2.12	61.28 ± 6.10	54.05 ± 3.51	36.54 ± 3.15	34.12 ± 3.54	40.72 ± 4.85
5 mg/kg E4 + 1.6 mg/kg P4	37.27 ± 3.44	34.00 ± 5.20	24.45 ± 5.83	52.05 ± 6.93	43.07 ± 3.66	36.96 ± 7.55	40.78 ± 9.06	44.91 ± 5.89
5 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	34.85 ± 2.10	36.95 ± 3.00	30.95 ± 3.64	53.75 ± 4.25	41.34 ± 2.37	35.08 ± 3.94	33.83 ± 5.17	45.44 ± 3.91

5 mg/kg E4 + 16 mg/kg P4	53.06 ± 2.55	43.67 ± 3.16	46.37 ± 3.61	47.08 ± 5.96	54.97 ± 3.07	49.78 ± 2.58	52.41 ± 4.25	45.84 ± 5.02
5 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	41.97 ± 2.02	43.27 ± 2.84	38.16 ± 3.91	39.14 ± 3.92	42.71 ± 3.02	44.08 ± 4.12	35.17 ± 3.88	34.58 ± 3.06
5 mg/kg E4 + 136 ng/kg E2	41.69 ± 2.75	54.47 ± 4.17	43.03 ± 5.13	53.37 ± 3.72	40.10 ± 4.40	50.32 ± 3.00	43.75 ± 3.83	36.50 ± 3.17
10 mg/kg E4	43.67 ± 2.26	41.08 ± 2.47	36.18 ± 3.70	53.88 ± 3.90	44.65 ± 3.38	46.16 ± 2.96	49.14 ± 3.84	46.66 ± 3.90
10 mg/kg E4 + 1.6 mg/kg P4	49.64 ± 4.35	45.286 ± 4.01	52.53 ± 5.02	44.61 ± 5.31	43.31 ± 3.97	51.91 ± 1.57	49.58 ± 4.25	41.96 ± 6.01
10 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	37.73 ± 3.38	44.74 ± 7.93	31.47 ± 6.98	47.19 ± 6.10	46.97 ± 3.91	39.40 ± 3.79	40.78 ± 4.16	34.81 ± 3.12
10 mg/kg E4 + 16 mg/kg P4	41.10 ± 4.00	48.83 ± 2.69	46.83 ± 3.53	62.66 ± 3.74	46.85 ± 3.19	50.72 ± 3.83	52.68 ± 3.94	48.70 ± 3.76
10 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	33.53 ± 1.70	30.11 ± 2.24	27.84 ± 4.31	42.81 ± 2.93	45.08 ± 1.75	40.39 ± 3.51	39.24 ± 4.96	37.98 ± 3.76
10 mg/kg E4 + 136 ng/kg E2	39.89 ± 3.20	38.39 ± 6.74	42.00 ± 5.22	46.20 ± 3.74	47.03 ± 3.82	44.97 ± 8.41	40.61 ± 6.51	39.07 ± 3.32

Significant differences were observed:

In the DG region

Pretreated groups: *DCX-stained cells*—[a]vehicle vs. sham, 5 mg/kg/day E4; *VEGF-stained cells*—[b]vehicle vs. sham, 5 mg/kg/day E4 + 16 mg/kg/day P4

Treated groups: *DCX-stained cells*—[c]vehicle vs. sham, 5 mg/kg/day E4, 5 mg/kg/day E4 + 16 mg/kg/day P4, [d]sham vs. 5 mg/kg/day E4 + 1.6 mg/kg/day P4, 5 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2; *VEGF-stained cells*—[e]vehicle vs. sham, 5 mg/kg/day E4, 5 mg/kg/day E4 + 16 mg/kg/day P4

In the CA1 region

Pretreated groups: DCX-stained cells—[f]vehicle vs. sham; *VEGF-stained cells*—[g]vehicle vs. sham, 5 mg/kg/day + 16 mg/kg/day P4

Treated groups: *DCX-stained cells*—[h]vehicle vs. sham, 5 mg/kg/day E4 + 136 ng/kg/day E2; *VEGF-stained cells*—[i]vehicle vs. sham, 10 mg/kg/day E4 + 1.6 mg/kg/day P4

In the CA2/CA3 region

Pretreated groups: *DCX-stained cells*—[j]vehicle vs. sham; *VEGF-stained cells*[k]vehicle vs. sham; [m]5 mg/kg/day E4 vs. sham, 5 mg/kg/day E4 + 16 mg/kg/day P4

Treated groups: *DCX-stained cells*—[l]vehicle vs. sham, 10 mg/kg/day + 1.6 mg/kg/day P4; [m]5 mg/kg/day E4 vs. sham, 5 mg/kg/day E4 + 16 mg/kg/day P4; *VEGF-stained cells*—[n]vehicle vs. sham

In the cortex

Pretreated groups: *DCX-stained cells*—[o]vehicle vs. sham, 5 mg/kg/day E4 + 16 mg/kg/day P4; *VEGF-stained cells*—[p]vehicle vs. sham, 5 mg/kg/day E4 + 16 mg/kg/day P4

Treated groups: *DCX-stained cells*—[q]vehicle vs. sham; *VEGF-stained cells*—[r]vehicle vs. sham

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treated by 5 mg/kg/day E4 alone or with 16 mg/kg/day P4 (Fig. 4.13). Also, sham group showed significantly higher number of DCX positively stained cells than the groups combinedly treated either by 5 mg/kg/day with 1.6 mg/kg/day P4 or 10 mg/kg/day E4 with 16 mg/kg/day P4 and E2 as well as groups treated by E4 in combination with 1.6 mg/kg/day P4 and E2 (Table 4.6) [117]. In the same region, angiogenesis was significantly upregulated in the sham-operated animals and in groups treated by 5 mg/kg/day E4 alone or in combination with 16 mg/kg/day P4; in the CA1 region, neurogenesis was significantly upregulated along with the sham group in animals treated by 5 mg/kg/day E4 along with E2 than in the vehicles, whereas angiogenesis was significantly upregulated in sham group and in animals treated by 10 mg/kg/day E4 with 1.6 mg/kg/day P4 (Table 4.6) [117]; In the CA2/CA3 region expressions of DCX and VEGF were significantly different between sham and the vehicle groups. In the CA2/CA3 region significant differences in DCX expression was detected between sham group, the animals treated by 10mg/kg/d E4 with 1.6mg/kg/d P4 and the vehicles as well as between sham group, the animals treated by 5mg/kg/d E4 with 16mg/kg/d P4 and the group treated by 5mg/kg/d E4 alone (Table 4.6); in the same region, VEGF was significantly more expressed in sham group than in the vehicles; in the cortex neuro- and angiogenesis were significantly more upregulated only in sham group compared to the vehicle group (Table 4.6); in general, combination of E4 with E2 resulted in low DCX and VEGF expression levels in the cortex (Fig. 4.13) [117].

The next step was evaluation of brain damage marker (GFAP) as it was discussed previously [117]. Combined pretreatment by 5 mg/kg/day E4 with P4 and E2 resulted in significant downregulation of GFAP expression compared to the vehicle group (Table 4.7). Different patterns of GFAP expression were observed in different groups. Significant downregulation of GFAP concentration was also observed in animals pretreated either by 5 mg/kg/day E4 alone or in combination with different doses of P4 and E2 or combined with 16 mg/kg/day P4 and in sham group than in animals pretreated by 5 mg/kg/day E4 and E2 (Table 4.7), also between groups pretreated by 10 mg/kg/day E4 alone or in combination with different doses of P4 and E2 or with 16 mg/kg/day P4 than in animals pretreated by 10 mg/kg/day E4 and E2 (Table 4.7) [117]. Treatment by E4 with P4 and/or E2 resulted in significant decrease of GFAP protein concentration in 10 mg/kg/day E4 along with the sham groups compared to animals treated by combination of 10 mg/kg/day E4 and E2 (Table 4.7). In vivo, in pretreated/treated groups, the combination of E4 and E2 showed significantly higher levels of GFAP, suggesting a negative cooperativity of these steroids upon cell survival (Table 4.7) [118].

After taking into account all the observations and experimental results, we have defined that combined use of E4 with other steroids has no benefit over the single use of E4 [117].

Table 4.7 Glial fibrillary acidic protein (GFAP) expression in blood serum (pg/ml) of the combinedly pretreated/treated rat pups

	Combined pretreatment		Combined treatment	
Group	pg/ml	*N* of samples	pg/ml	*N* of samples
Sham	2393.40 ± 1454.429[a]	8	2393.40 ± 1454.43[e]	8
Vehicle	23,915.91 ± 3158.84[b]	10	28,901.155 ± 4480.30	11
5 mg/kg E4	6220.49 ± 1763.17	11	6380.10 ± 4062.591	10
5 mg/kg E4 + 1.6 mg/kg P4	12,548.31 ± 2280.50	10	8146.34 ± 3596.07	10
5 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	1011.42 ± 55.32	13	19,226.69 ± 2559.70	10
5 mg/kg E4 + 16 mg/kg P4	5113.67 ± 1733.57	10	25,919.72 ± 4487.50	10
5 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	737.01 ± 69.82	11	17,476.73 ± 2643.53	10
5 mg/kg E4 + 136 ng/kg E2	28,442.46 ± 3457.11[c]	11	32,354.42 ± 5946.66	10
10 mg/kg E4	12,413.45 ± 2243.05	12	10,806.52 ± 1915.19[f]	10
10 mg/kg E4 + 1.6 mg/kg P4	27,225.88 ± 8442.88	7	18,796.20 ± 4279.45	10
10 mg/kg E4 + 1.6 mg/kg P4 + 136 ng/kg E2	9672.46 ± 2461.11	12	20,470.58 ± 1468.47	14
10 mg/kg E4 + 16 mg/kg P4	12,037.18 ± 3726.66	12	15,974.26 ± 2111.42	11
10 mg/kg E4 + 16 mg/kg P4 + 136 ng/kg E2	11,202.39 ± 2765.16	11	22,202.18 ± 2624.61	11
10 mg/kg E4 + 136 ng/kg E2	32,898.22 ± 3437.25[d]	11	26,660.81 ± 4870.81	10

Significant differences were observed:
In pretreated groups: [a]sham vs. vehicle, 10 mg/kg/day E4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 1.6 mg/kg/day P4; [b]vehicle vs. 5 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 5 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2; [c]5 mg/kg/day E4 + 136 ng/kg/day E2 vs. sham, 5 mg/kg/day E4, 5 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 5 mg/kg/day E4 + 16 mg/kg/day P4, 5 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2; [d]10 mg/kg/day E4 + 136 ng/kg/day vs. sham, 10 mg/kg/day E4, 10 mg/kg/day E4 + 1.6 mg/kg/day P4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 16 mg/kg/day P4, 10 mg/kg/day E4 + 16 mg/kg/day P4 + 136 ng/kg/day E2
In treated groups: [e]sham vs. vehicle, 5 mg/kg/day E4 + 136 ng/kg/day E2, 10 mg/kg/day E4 + 136 ng/kg/day E2; [f]10 mg/kg/day E4 vs. 10 mg/kg/day E4 + 136 ng/kg/day E2
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4.4.3 How E4 Is Realizing Its Neuroprotective Effects?

Recent studies in different cells and tissues showed that E4 acts as a selective estrogen receptor modulator (SERM) by activating the nuclear ERα, inhibiting its membrane form and blocking the membrane initiated steroid signaling by E2 [149]. E4 may have a synergistic role with E2 (through activation of nuclear ERα) or an

antiestrogenic effect by blocking membrane ERα and its activation by E2 depending on the respective role of nuclear and membrane forms of ERα in target organs. Thus, E4 has biological activities distinct from E2, depending on the tissues and cells and the selective binding to the nuclear/membrane form of ERα [149]. In general, palmitoylation regulates 17β-estradiol-induced ERα degradation and transcriptional activity [150] and may explain the ability of ERα to associate to plasma membrane making possible E2-dependent rapid functions [151], and the same might be plausible for E4-dependent rapid functions. Recent studies also have shown that ERβ expression in oligodendrocytes is important for the attenuation of clinical disease by an ERβ ligand, like that pointing an importance of this receptor in myelination [152].

As far we were going in our research as meticulously, we were trying to identify the exact mechanism of E4-dependent neuroprotective actions. Recent studies showed that, in general, the neuroprotective actions of estrogens among other factors also depend on their strong antioxidant properties. All estrogens have a phenolic moiety in their structure, the free phenolic OH group, which has been considered the quintessential feature in conferring protection against oxidative stress [153]. E4 has the highest number (four) of free phenolic hydroxyl groups in its structure, thus pointing out the possibility to have stronger antioxidant properties than other estrogens. Thus, one more explanation for E4 neuroprotective effect might be attributed to its strong antioxidant effect as well, which is demonstrated by our previous studies [116, 117], but it is not enough to explain the full spectrum of impressive results of action of E4 in the CNS.

For in vitro studies, we have used one of the most successful concentrations of E4 (3.25 mM) already defined from our previous research [116] alone or in combination with different estrogen receptor inhibitors and/or palmitoylation inhibitor after induction of oxidative stress in primary hippocampal neuronal cell cultures. The antioxidative activity of E4 and the expression of LDH were completely blocked only by concomitant treatment of cells with E4, MPP (inhibitor of ERα), and PHTTP (inhibitor of ERβ) (Fig. 4.14a, b) [118]. Inhibition of palmitoylation alone with 2-BR or in combination with MPP significantly decreased LDH activity, suggesting that the combined blockage of ERα and palmitoylation is not sufficient to inhibit the E4-dependent effects (Fig. 4.14c), whereas combination of E4 with 2-BR, MPP, and PHTTP completely blocked the antioxidative effects of E4 once again suggesting the role of both receptors, ERα and ERβ (Fig. 4.14c). Inhibition of GPR30 receptor did not block the E4 actions (Fig. 4.14d) [118].

Cell survival rate was significantly downregulated only by inhibition of ERβ alone (Fig. 4.15b). All cells treated either by E4 alone or in combination with different combinations of 2-BR, MPP, and PHTTP had significantly higher cell survival rate, and inhibition of palmitoylation along with inhibition of ERα activity resulted in a significantly higher cell survival rate suggesting that ERα (probably membrane form of the receptor) does not affect the E4-dependent cell survival/proliferation actions (Fig. 4.15c). Inhibition of GPER did not affect the cell survival rate (Fig. 4.15d) [118].

As we have already discussed earlier, the expression of ERα and ERβ displays different spatial-temporal patterns during human cortical and hippocampal

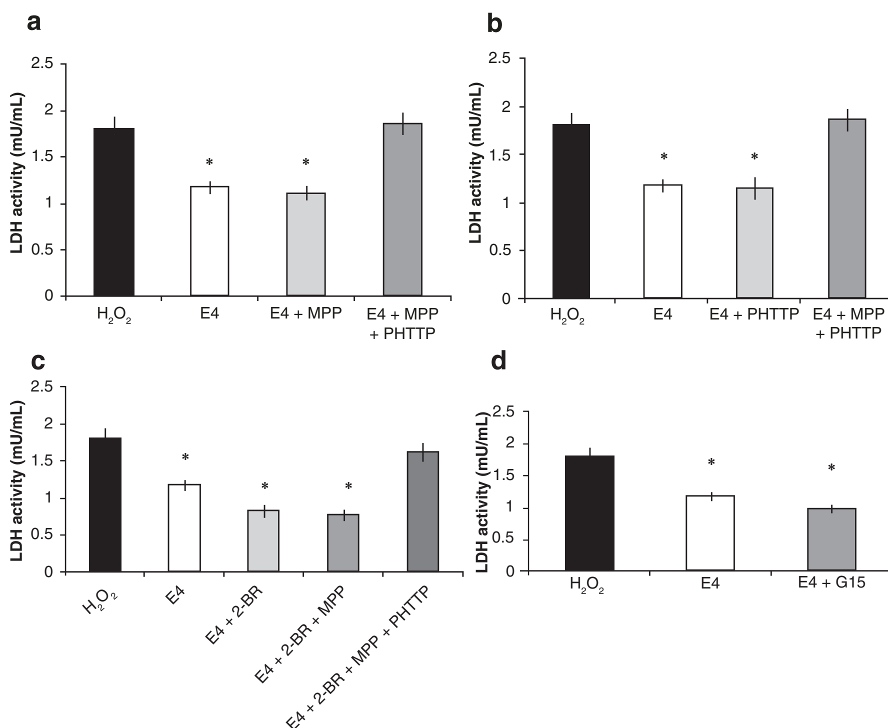

Fig. 4.14 Effect of E4 in combination with different receptor inhibitors on LDH activity in primary hippocampal neuronal cultures subjected to the H_2O_2-induced oxidative stress. Primary hippocampal cell cultures were exposed to 3.25 mM E4 alone or in combination with MPP, PHTTP, G15, and/or 2-BR after induction of oxidative stress. (**a**) LDH activity was significantly decreased by treatment with E4 alone or in combination with ERα inhibitor MPP compared to the H_2O_2-treated cell cultures or cultures combinedly treated by E4 + MPP + PHTTP. Combined use of MPP and PHTTP significantly increased the LDH activity compared to the cells treated by E4 alone or in combination with MPP. (**b**) LDH activity was significantly decreased by treatment with E4 alone or in combination with ERβ inhibitor PHTTP compared to the H_2O_2-treated cell cultures or cultures combinedly treated by E4 + MPP + PHTTP. Combined use of MPP and PHTTP significantly increased the LDH compared to the cell cultures treated by E4 alone or in combination with PHTTP. (**c**) Inhibition of palmitoylation alone or in combination with MPP significantly downregulated LDH activity compared to the H_2O_2-treated cells or to those treated by E4 alone. Combination of E4 with 2-BR, MPP, and PHTTP significantly upregulated LDH activity compared to the cell cultures treated by E4 or 2-BR alone or in combination with MPP. (**d**) Cell cultures treated by E4 alone or in combination with GPR30 inhibitor G15 had significantly lower LDH activity compared to the cultures treated by H_2O_2 alone. No significant difference was observed between the cells treated by E4 alone or in combination with G15. Reproduced from J Endocrinol (Tskitishvili et al., 2017, 232(1):85–95)

development, and knowledge of the region-specific expression of each ER subtype is critical to better understand the actions of estrogens on the human brain [3]. Even though, the genomic effects of ERs are mostly studied, we have to pay attention to the rapid cellular signaling (non-genomic) effects that are thought to be mediated primarily by membrane-associated forms of these receptors [154]. These

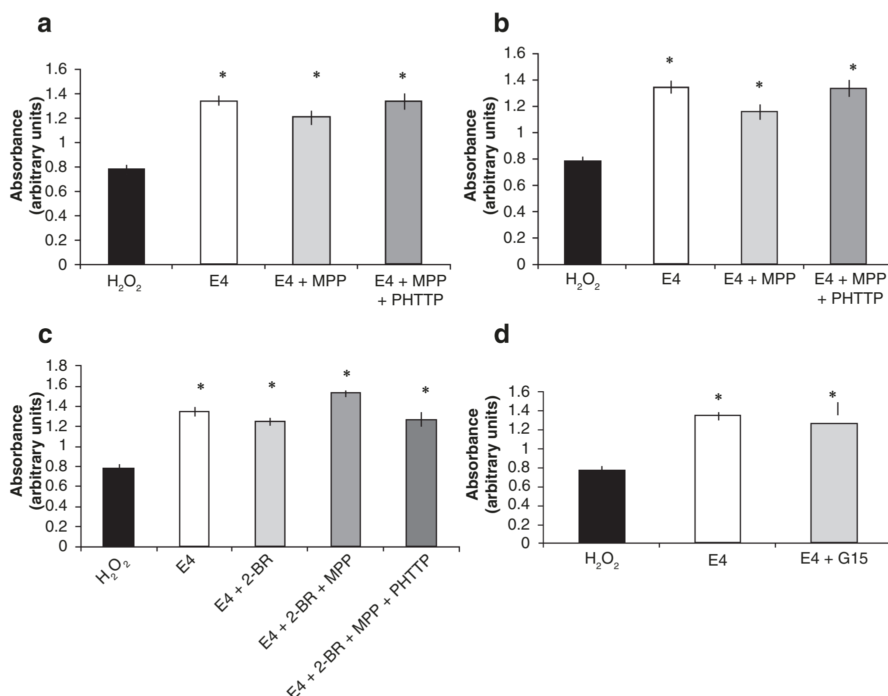

Fig. 4.15 Effect of E4 in combination with different receptor inhibitors on cell survival in primary hippocampal neuronal cultures subjected to the H_2O_2-induced oxidative stress. Primary hippocampal cell cultures were exposed to 3.25 mM E4 alone or in combination with MPP, PHTTP, G15, and/or 2-BR after induction of oxidative stress. (**a**) Cell survival rate was significantly upregulated in cells treated by E4 alone or in combination either with MPP or MPP + PHTTP compared to cells solely treated by H_2O_2. (**b**) Cultures treated either by E4 alone or with PHTTP with/without MPP had significantly upregulated cell survival rate compared to cells treated by H_2O_2 alone. Cells combinedly treated by E4 with PHTTP had significantly lower cell survival rate than the cell cultures treated by E4 alone. (**c**) Cells treated either by E4 alone or in combination with 2-BR, MPP, and/or PHTTP had significantly higher cell survival rate compared to the cells solely treated by H_2O_2. Treatment of cultures by E4 and 2-BR along with MPP resulted in significant upregulation of cell survival compared to the cultures treated by 2-BR alone or in combination with MPP and PHTTP. No significant difference was observed between the cells treated by E4 alone or those treated by different combinations of E4, 2-BR, MPP, and/or PHTTP. (**d**) Treatment of cell cultures by E4 alone or in combination with G15 significantly upregulated the cell survival rate compared to cell cultures treated by H_2O_2. No significant difference was observed between cells treated by E4 alone or in combination with G15. Reproduced from J Endocrinol (Tskitishvili et al., 2017, 232(1):85–95)

non-genomic signaling events are dependent to the estrogen-binding sites on intracellular membranes, whereas some reports suggest that palmitoylation or phosphorylation [150] may target classical ERs to the cytoplasmic side of the plasma membrane. In general, palmitoylation is necessary for ERα transcriptional activity, and inhibition of ERα palmitoylation constitutively addresses ERα to the nuclear matrix resulting in the basal degradation of the neo-synthesized ERα [155], though

we did not observe any significant effect of palmitoylation inhibition on cell survival/proliferation [118].

It was already defined that the potential role of ERβ expression in cells of oligodendrocyte (OL) lineage in ERβ ligand-mediated neuroprotection is important, and it results in the upregulation of myelination [152]. Also, neuroprotection might be mediated through ERα in astrocytes exclusively [156]. Our studies prove that the E4-mediated activities in the CNS are realized through ERα and ERβ, like that enlightening the important role of E4 as a selective estrogen receptor modulator (SERM) with neurosteroid actions.

4.5 Conclusion

Summarizing our findings we can admit that for the first time we proved impressive antioxidative, neuroprotective, promyelinating, and neuro-, and angiogenesis effects of estetrol in the CNS by employing in vitro and in vivo studies. We believe that our investigation will open new horizons for the development of new perinatal treatment strategies not only for HIE but for periventricular leukomalacia (PVL) as well. Our research could also contribute to a better understanding of the pathophysiology and treatment of other neurological diseases such as Alzheimer's and Parkinson's diseases, traumatic brain injury, and multiple sclerosis.

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