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Reversion by Vitamin E Treatment of the Oxidative Damage but Not of the Advancement in Reproductive Senescence Produced by Neonatal Hypoxia or Hypoxia-Ischemia in Female Rats

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Key Words
Senescence · Reproduction · Hormone receptors · Female rat · Hypoxia/ischemia · Oxidative damage

Abstract
Background/Aims: Few studies address the long-term consequences of perinatal hypoxia (H), a frequent birth complication. Previously we described advanced reproductive senescence (premature loss of regular cyclicity) in female rats subjected to perinatal H or H plus unilateral ischemia (HI) associated with changes in the hypothalamic expression of estrogen and opioid receptors. Our aim is to explore whether hypothalamic inflammation and oxidative damage mediate these reproductive alterations. ***Methods:*** Female rats were subjected on postnatal day (PND) 7 to H (6.5% O_2 for 50 min) or HI (H + right carotid artery ligature) and inflammation/oxidative damage markers, such as iNOS, nNOS, insulin-like growth factor (IGF) system expression, glial reaction and macrophage invasion in the medial basal hypothalamus-preoptic area (GFAP Western blot and immunohistochemistry, ED1 immunohistochemistry), were determined. The effect of antioxidant treatment with vitamin E (VE; 1.5 mg/rat on PND 4, 6 and 8) was also explored. ***Results:*** No significant cellular inflammatory reactions were observed although GFAP protein was significantly increased at early times after injury. Forty-eight hours after injury iNOS, nNOS and IGF-I mRNA decreased in the HI group, and nNOS in the H group. IGFBP-3 mRNA increased in HI rats at 48 h and 30 days, while it fell at 7 days postinjury in both groups. VE treatment prevented the effects of HI on oxidation/inflammation markers, but did not prevent the premature onset of reproductive senescence or the altered hormone receptors expression. ***Conclusion:*** These results suggest that the oxidative and inflammatory damage caused by perinatal H or HI may not be responsible for the late-onset reproductive abnormalities.

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Introduction

Although the effects of neonatal hypoxia (H) or hypoxia-ischemia have been well studied, and different therapeutic approaches designed to limit damage have been explored, there are very few studies concerning the long-term consequences of a hypoxic episode near birth. In our previous study [1], we described a significantly premature onset of reproductive senescence (evidenced by a 3–4 month earlier start of cycling irregularities and the establishment of anestrus) in female Sprague-Dawley

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rats subjected to mild H or hypoxia plus unilateral ischemia (HI). These changes were associated with early decreases in the expression of estrogen receptor (ER), progesterone receptor (PR) and opioid receptor (OR) in the mediobasal hypothalamus-preoptic area (MBH-PO), followed by increased μOR protein and ERβ mRNA in the H group and ERα, ERβ and μOR mRNAs and ER protein in the HI group 30 days postinjury [1]. Altered expression of these proteins has been associated with aging of the reproductive system [2–4], among other factors.

On the other hand, the endocrine changes associated with reproductive senescence are complex and may not be solely associated with simple changes in the concentration of hormones or their receptors in relevant hypothalamic areas. It is quite probable that other factors indirectly associated with hormone action may contribute to the early reproductive senescence phenotype and cannot be ignored if we wish to elucidate the mechanism of this process. For example, chronic oxidative damage has been implicated in the aging process through the production of reactive oxygen species (ROS) that in turn produce lipid peroxidation and damage to tissues, in particular lipid-rich ones, such as the central nervous system. Thus, oxidative stress mimics the effects of aging on cognitive impairment and antioxidant treatment with vitamin E (VE; α-tocopherol) can prevent or delay the damage [5–7]. At least part of the brain tissue damage produced by H or HI may be caused by oxidative stress that originated in the reperfusion process [8, 9]. Inhibition of these processes prevents edema and neuronal damage [9–11]. For this reason, antioxidants have been used for neuronal protection in different experimental models of neuronal damage.

In particular, oxidative damage produced by the repeated exposure to elevated estradiol caused by the preovulatory peaks occurring during the reproductive period have been implied as a cause of reproductive senescence, rather than aging per se [12]. In support of this hypothesis, the damage produced by chronic exposure to estrogens can be mitigated by free radical scavengers, such as vitamins C and E, and glutathione. In fact, pharmacological actions of administered estrogen in causing premature constant estrus are antagonized by the administration of VE [13, 14]. VE has antioxidant and plasma membrane stabilizing properties [9, 15–17]. Pretreatment with VE reduces brain edema, promotes ATP synthesis and inhibits the neuronal signals produced after an HI episode [9, 10]. Furthermore, VE deficiency produces neurological defects that are similar to those caused by normal aging [8, 18].

Nitric oxide (NO) has an important role in the mechanism of H through its vasodilatory action, and it may also contribute to the injury when an excessive amount produced during reperfusion can induce oxidative damage [8, 9]. NO has also been implicated in the regulation of sex behavior [19] and in the secretion of various pituitary hormones [20–22], while brain NO activity is modified by stimuli that also affect hormone secretion, such as gonadectomy [23], lactation [24] and stress [25].

The insulin-like growth factor (IGF) system has also been implicated in the regulation of the hypothalamo-pituitary-gonadal axis, for example during puberty [26, 27]. It regulates the neurotrophic response after HI [28], brain glucose consumption [29] and brain neurogenesis [30, 31]. Reduced circulating and brain IGF-I levels have been associated with physiopathological changes linked to aging [32].

The purpose of the present work is to contribute to the elucidation of the hypothalamic mechanisms through which neonatal HI or H advance reproductive senescence, in particular whether the effects are mediated by oxidative damage. With this aim we determined the effects of HI or H on different markers of inflammation and oxidative damage, such as: (a) the expression of iNOS and nNOS in the MBH-PO, since these molecules are markers of oxidative damage and are relevant for the regulation of the hypothalamic pituitary gonadal axis, (b) the expression of the main components of the IGF system in the MBH-PO, (c) the development of a glial reaction and macrophage invasion in the MBH-PO in response to the injuries. We also explored whether an antioxidant treatment with VE is able to prevent premature reproductive senescence and the changes in estrogen, progesterone, opioid receptors and inflammation markers produced by HI.

Methods

Animals

Female Sprague-Dawley rats bred in our laboratory were kept in a temperature (23 ± 1°C) and light-dark cycle (lights on: 06.00–20.00 h) controlled environment and fed on a standard laboratory diet (Cargill, Cordoba, Argentina) and tap water ad libitum. The rats were mated on the night of proestrus and the following day was counted as day 0 of pregnancy if spermatozoa were observed in the vaginal smear. The rats were caged individually 2 or 3 days before the expected delivery. Delivery was allowed to proceed normally and the day of birth was defined as postnatal day (PND) 0. On day 1 of lactation, the number of pups of each litter was adjusted to 8 (by culling of surplus males) to insure adequate and similar nursing and maternal care. The pups were examined daily

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for general good health and otherwise left undisturbed with their mother until taken for surgery. Female pups were subjected to H or HI at PND 7, as described below. After these procedures, the surviving pups were returned to their mothers and remained with them until weaning at 22–23 days of age.

All the procedures performed on the animals were in accordance with the guidelines suggested by the NIH guide for the care and use of laboratory animals (NIH publication No. 86-23, revised 1985 and 1991) and the UK requirements for ethics of animal experimentation [the Animals (Scientific Procedures) Act 1986]. The experiments were approved by the institutional ethics committee.

Induction of Cerebral H

Induction of H was performed as previously described [33–35]. At PND 7, half of the female pups from each litter were placed in a 500-ml airtight chamber with controlled humidity and temperature (36.5°C) by partial submersion in a 37°C water bath to keep the pups warm during the procedure. A humidified gas mixture of 6.5% O_2 93.5% N_2 was delivered into the jar via inlet and outlet portals at an approximate flow rate of 100 ml/min. The pups were exposed to the gas mixture for 50 min. This procedure produced a mortality rate of less than 5%. Temperature was monitored during the H period. After H, the surviving pups (H) were allowed to recover for 15 min in the same jar in the warm water bath, but with the lid open. The remaining littermates (control group; CR) were placed in the jar for 50 min and maintained at the same warm temperature, breathing room air.

Unilateral Cerebral HI Model

We used the method described by Trescher et al. [36], with some modifications. Briefly, PND 7 female pups were separated from their mothers and kept warm, as described above. Under sevoflurane anesthesia, the right common carotid artery was isolated, ligated in two places and cut between the ligatures. The total time of surgery was about 7–10 min. After the surgical procedure, the animals were placed in the temperature-controlled container to recover for 40 min and were then exposed to 50 min of hypoxia (HI). H was induced as described above. CR animals comprised littermates of the treated pups subjected to sham surgery and placed in the warm jar breathing room air for 50 min. The mortality rate for the unilateral carotid artery ligature was approximately 10%.

VE Treatment

Female rat pups were injected s.c. with 1.5 mg of VE (α-tocopherol acetate) (Laboratorios Raymon SAIC, Buenos Aires, Argentina) in 50 μl of corn oil on PND 4, 6 and 8. CR rats were injected with the same volume of vehicle.

Evaluation of Growth, Sexual Maturation and Estrous Cycle

The pups were inspected daily to determine the age of hair and teeth irruption and of eye opening. To determine growth rates, the pups were weighed weekly and the timing of vaginal opening for female rats was assessed by daily visual inspection from day 30 onwards. Vaginal opening reflects the peripubertal estradiol secretion and has been used as the landmark for the progress of reproductive maturity. Immediately after vaginal opening, ovarian cycles were assessed by examination of daily vaginal smears, taken approximately at the same time each morning for 20 consecutive days of each month. Animals with two or more consecutive 4- to 5-day cycles were defined as having regular estrous cycles. Those with two or more consecutive 6 or more day cycles were defined as having irregular estrous cycles. Animals showing 14 days or more of cornified vaginal smears or sequences of several (3–5) days of cornified cell smears interspersed with 1 day of dioestrus smears were defined as persistent estrus and those with 10 or more days of diestrus smears were defined as persistent diestrus.

Determination of Hormone Concentrations

In order to determine the pattern of preovulatory hormonal secretion during the cycle, 8–10 regularly cycling rats of each experimental group, at 3 months of age, were bled by the tail vein under sevoflurane anesthesia twice (at 12.00 and 19.00 h) on proestrus and estrus days. The blood samples were allowed to clot at room temperature and the serum separated by centrifugation and stored at –30°C until used. Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were measured by double antibody radioimmunoassay, using materials provided by Dr. A.F. Parlow and the NHPP (National Hormone and Pituitary Program, Harbor-UCLA Medical Center, Torrance, Calif., USA). The hormones were radioiodinated using the chloramine T method and purified by passage through Sephadex G75. The results were expressed in terms of the rat LH RP-3 or FSH RP-3 standard preparations. Assay sensitivity was 0.5 μg/l of serum and the inter- and intra-assay coefficients of variation were less than 10% for all hormones.

Brain Tissue Sampling

At 48 h, 7 and 30 days, and 8 and 18 months after injury, groups of 12 (6 for RNA preparation and 6 for protein homogenate preparation) CR, H or HI rats were sacrificed by decapitation, the brains were rapidly removed and cut into 200-μm-thick coronal sections on a slicer (Rodent Brain Matrix RBM-400C, ASI Instruments Inc., Houston, Tex., USA). The sections were collected onto glass microscope slides and immediately frozen. A block of tissue comprising the arcuate nucleus, anteroventricular paraventricular nucleus, median eminence and preoptic area (MBH-PO) was microdissected and processed for RNA or total protein extraction.

Immunohistochemistry

At 7 and 30 days postinjury, groups of 3 CR, H or HI rats were deeply anesthetized with chloral hydrate and perfused intracardially with washing solution (0.5% NaCl, 0.8% sucrose and 0.4% glucose for 10 min) and then with 4% paraformaldehyde in 0.01 M borate buffer, pH 7.4, plus 0.35 M Na_2SO_3. The whole heads were wrapped in aluminum foil and stored overnight at 4°C for complete fixation. On the following day the brains were removed and cryoprotected with 30% sucrose in 0.01 M borate buffer, pH 7.4. After 2–3 days, the brains were rapidly frozen at –20°C in isopentane, kept in dry ice, and serially sectioned (30 μm) with a cryostat. Four sections containing the MBH (planes equivalent to plates 47–52 for the adult rat, described by Paxinos and Watson [37]) were selected for immunostaining. Slices were washed in 50 mM Tris-HCl, NaCl (0.9%), pH 7.4, reacted with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase, and then incubated for 45 min in 10% goat serum in blocking solution (saline/Tris buffer with 0.1% Triton X-100, 1% goat serum and 1% bovine serum albumin). After additional washes, free-floating sections were incubated with anti-GFAP (goat polyclonal C-19, 1/200; Santa Cruz Biotechnology Inc., Santa Cruz, Calif., USA), anti-ED1 (mouse

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Table 1. Primer sequences and reaction conditions used in the PCR amplification of the various cDNAs

cDNA	Primers 5′–3′	Cycles, n	Quantity of cDNA added, ng	Product	Gene bank accession
IGF-I S IGF-I AS	AAAATCAGCAGTCTTCCAAC AGATCACAGCTCCGGAAGCA	30	50	299 pb	X06108
IGFBP-3 S IGFBP-3 AS	GCCGCGGGCTCTGCGTCAACGC CTGGGACTCAGCACATTGAGGAAC	25	50	415 pb	NM012588
IGFBP-5 S IGFBP-5 AS	TTGCCTCAACGAAAAGAGC AGAATCCTTTGCGGTCACA	25	50	377 pb	NM012817
IGF-IR S IGF-IR AS	TCCACCATAGACTGGTCTCT ACGAAGCCATCTGAGTCACT	30	50	433 pb	L29232
iNOS S iNOS AS	GCATGGACCAGTATAAGGCAAGCA GCTTCTGGTCGATGTCATGAGCAA	30	50	219 pb	S71597
nNOS S nNOS AS	GAACCCCCAAGACCATCC GGCTTTGCTCCCACAGTT	30	50	308 pb	X59949
TNFα S TNFα AS	CTCGAGTGACAAGCCCGTAG TTGACCTCAGCGCTGAGCAG	30	50	387 pb	AF329985
ERα S ERα AS	AATTCTGACAATCGACGCCAG GTGCTTCAACATTCTCCCTCCTC	30	100	345 bp	NM012689
ERβ S ERβ AS	AAAGCCAAGAGAAACGGTGGGCAT GCCAATCATCTGCACCAGTTCCTT	25	100	204 bp	NM012754
μOR S μOR AS	ACCTGGCTCCTGGCTCAACTT TGGACCCCTGCCTGTATTTTG	25	30	569 bp	NM013071
PR S PR AS	CCCACAGGAGTTTGTCAAGCTC TAACTTCAGACATCATTTCCGG	30	50	325 bp	NM0228471
β-Actin S β-Actin AS	CGTGGGCCGCCCTAGGCACCA TTGGCCTTAGGGTTCAGAGGGG	25	30	243 bp	BC063166

All reactions were carried out with the following cyclic parameters: 95°C for 1 min, 62°C for 1 min and 72°C for 1 min, except that the annealing temperature was 57°C for iNOS and nNOS, and 59°C for TNFα. All the reactions were terminated with a 5 min extension at 72°C.

anti-rat monoclonal CD68 Clone ED1, 1/200; Serotec, Kidlington, UK) and biotynilated anti-goat γ-globulin 1/500 (Vector Laboratories, Burlingame, Calif., USA) or biotynilated anti-mouse γ-globulin, 1/500, (Vector Laboratories) as secondary antibodies. The Vectastain avidin-peroxidase kit (Vector Laboratories) was used as a detector. Diaminobenzidine, glucose oxidase (Sigma, St. Louis, Mo., USA), plus nickel ammonium sulfate (as color enhancer) were used as a chromogen. Slices were mounted, dehydrated, and coverslipped with synthetic Canada balsam (Biopack, Buenos Aires, Argentina).

RNA Extraction and Reverse Transcription PCR

Total RNA was isolated from microdissected MBH-PO from CR, H and HI rats with TriZol (Gibco-BRL, Life Technologies, Carlsbad, Calif., USA), according to the manufacturer′s instructions. Integrity of the isolated total RNA was examined by 1% agarose gel electrophoresis, and RNA concentration was determined by the ultraviolet light absorbance at 260 nm. Reverse transcription (RT) was carried out using 10 μg of total RNA from the MBH-PO of each rat. RT was performed at 37°C for 60 min using 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). Aliquots of the reverse transcription reaction were amplified with specific primers (table 1). For the PCR amplification, specific oligonucleotide primers (0.5 μM each) were incubated with 5 μl of cDNA template in a 35-μl PCR reaction mixture containing 1.5 mM of $MgCl_2$, 25 mM of KCl, 10 mM of Tris-HCl, pH 9, 1 μl of deoxynucleotides (1 mM each) and 1 unit of Taq polymerase (Invitrogen, Carlsbad, Calif., USA). All the products were analyzed in the linear range of the RT-PCR amplification process (see table 1 for specific conditions of each amplification). RNA samples were assayed for DNA contamination by PCR without the prior reverse transcription. The products of each reaction were separated on 1.5% agarose gels containing 0.5 mg/ml of ethidium bromide and photographed with a digital camera. Band intensities of RT-PCR

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products were quantified using NIH Image software; relative levels of mRNA were calculated as the ratio of signal intensity for the target genes relative to β-actin cDNA. The results are expressed as arbitrary OD units.

Western Blot

For Western blots the tissues were placed in buffer (0.1 M of NaCl, 0.01 M of Tris-HCl, pH 7.6, plus 0.001 M of EDTA, pH 8, with a protease inhibitors mix consisting of 1 mM of PMSF, 2 μg/ml of aprotinin, 2 μg/ml of leupeptin and 2 μg/ml of pepstatin A) and homogenized. Aliquots were removed to determine protein content, and the remaining tissue was denatured with SDS (1% final concentration) and β-mercaptoethanol (1% final concentration) and stored at −70°C until the assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12%) was performed as described previously [1]. In brief, 10 μg of protein were loaded per lane. Molecular weight markers were run in parallel. Electrophoresis was performed at 100 V for 90 min and electrotransferred overnight (~16 h) at 30 V onto 0.2-μm-diameter pore nitrocellulose membranes (Hybond™ C; Amersham Life Science, Little Chalfont, UK). Then, the membranes were rinsed in PBS buffer containing 0.1% Tween 20 (PBS-T), pH 7.4, blocked for 2 h with 3% BSA and 2% horse serum (Sigma) in PBS-T and incubated for 2 h at room temperature in primary serum anti-GFAP (goat polyclonal C-19, 1/1,000, Santa Cruz Biotechnology Inc.) in blocking solution. After three washes in PBS-T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antisera (1/10,000 monkey anti-goat; Sigma) in blocking solution. After three washes in PBS-T, specific receptor bands were detected by chemiluminescence (ECL™; Amersham Life Science) using a ChemiDoc XRS+ system with Image Lab Software from Bio-Rad (Hercules, Calif., USA) and then quantified by densitometry using digital image processing by the NIH Image 1.6/ppc freeware program.

Statistical Analysis

For each set of values, the mean and standard error of the mean were calculated and compared using one- or two-way analysis of variance (ANOVA) with the statistical computer analysis system GraphPad Prism. When ANOVA revealed statistical differences, we applied the τ statistical post hoc analysis. Differences between means were considered significant at the $p < 0.05$ level.

Results

Effects of H or HI on Astrocyte and Macrophage Activation in Brain and MBH-PO

In earlier work, we have shown that perinatal HI induces glia activation in specific brain areas [33, 38] using immunohistochemistry of GFAP (as a specific marker of astrocytes, [39]) and of ED1 (an extensively used marker for activated macrophages in the rat expressed in cytoplasmic granule membranes [40, 41]). Confirming our previous results, in the present work we also found a profuse glial reaction on the brain areas directly affected by the ischemia, such as the somatosensory cerebral cortex, the striatum and the hippocampus in the ipsilateral side to the carotid ligature. At 30 days postlesion we detected strong GFAP+ and ED1+ signals in the ipsilateral somatosensory cortex (not shown). In the same trend, the brains of the H animals showed an enhanced bilateral glial response due to the global hypoxia (not shown). These responses were observed within the first week after injury and persisted throughout the postnatal developmental period.

In the hypothalamus, we did not observe the presence of macrophages at any of the times studied in either experimental group (H and HI). The presence of few GFAP+ reactive astrocytes in the MBH-PO was confirmed by Western blot analysis, a more sensitive method that showed significant increases in both injured groups (H and HI) at 48 h and in the HI group at 7 days (fig. 1d–f), while there were no significant differences between the three groups at 30 days. These results show a transient increase in the hypothalamic content of the intermediate filament protein at early times after injury [39].

Effects of H or HI on MBH-PO Oxidative Stress Markers and IGF System Proteins

We measured by RT-PCR the acute (at 48 h and 7 days postinjury) and delayed (at 30 days, 8 and 18 months postinjury) effects of H or HI on the expression of factors related with responses to oxidative damage, such as iNOS and nNOS and members of the IGF family. Most of the mRNAs measured showed significant changes with time (fig. 2, 3) and the effects of H or HI, appeared as expected, mostly at early times (48 h or 7 days) after injury (two-way ANOVAs, iNOS: $F_{time, 4, 71} = 44.97$, $p < 0.0001$; $F_{lesions, 2, 71} = 1.523$, $p = 0.225$; $F_{interaction, 8, 71} = 2.288$, $p = 0.0306$; nNOS: $F_{time, 4, 70} = 69.09$, $p < 0.0001$; $F_{lesions, 2, 70} = 0.4936$, $p = 0.6125$; $F_{interaction, 8, 70} = 3.014$, $p = 0.0058$; IGF-I: $F_{time, 4, 69} = 7.448$, $p < 0.0001$; $F_{lesions, 2, 69} = 2.206$, $p = 0.1178$; $F_{interaction, 8, 69} = 3.100$, $p = 0.0048$; IGF-IR: $F_{time, 4, 69} = 47.38$, $p < 0.0001$; $F_{lesions, 2, 69} = 2.576$, $p = 0.0834$; $F_{interaction, 8, 69} = 0.9982$, $p = 0.4455$; IGFBP-3: $F_{time, 4, 70} = 12.57$, $p < 0.0001$; $F_{lesions, 2, 70} = 0.08899$, $p = 0.9150$; $F_{interaction, 8, 70} = 3.854$, $p = 0.0010$; IGFBP-5: $F_{time, 4, 70} = 23.31$, $p < 0.0001$; $F_{lesions, 2, 70} = 15.09$, $p < 0.0001$; $F_{interaction, 8, 70} = 0.6899$, $p = 0.6988$).

Thus, the mRNA abundance of iNOS and nNOS were significantly decreased in MBH-PO 48 h after injury in HI rats, while in H rats only nNOS was decreased (fig. 2). There were no changes at later times, 7 days or 1, 8 and 18 months postinjury, with the exception of the HI group at 30 days, in which nNOS was significantly increased compared with the CR group. In CR rats we also found significant decreases in nNOS expression at 30 days and

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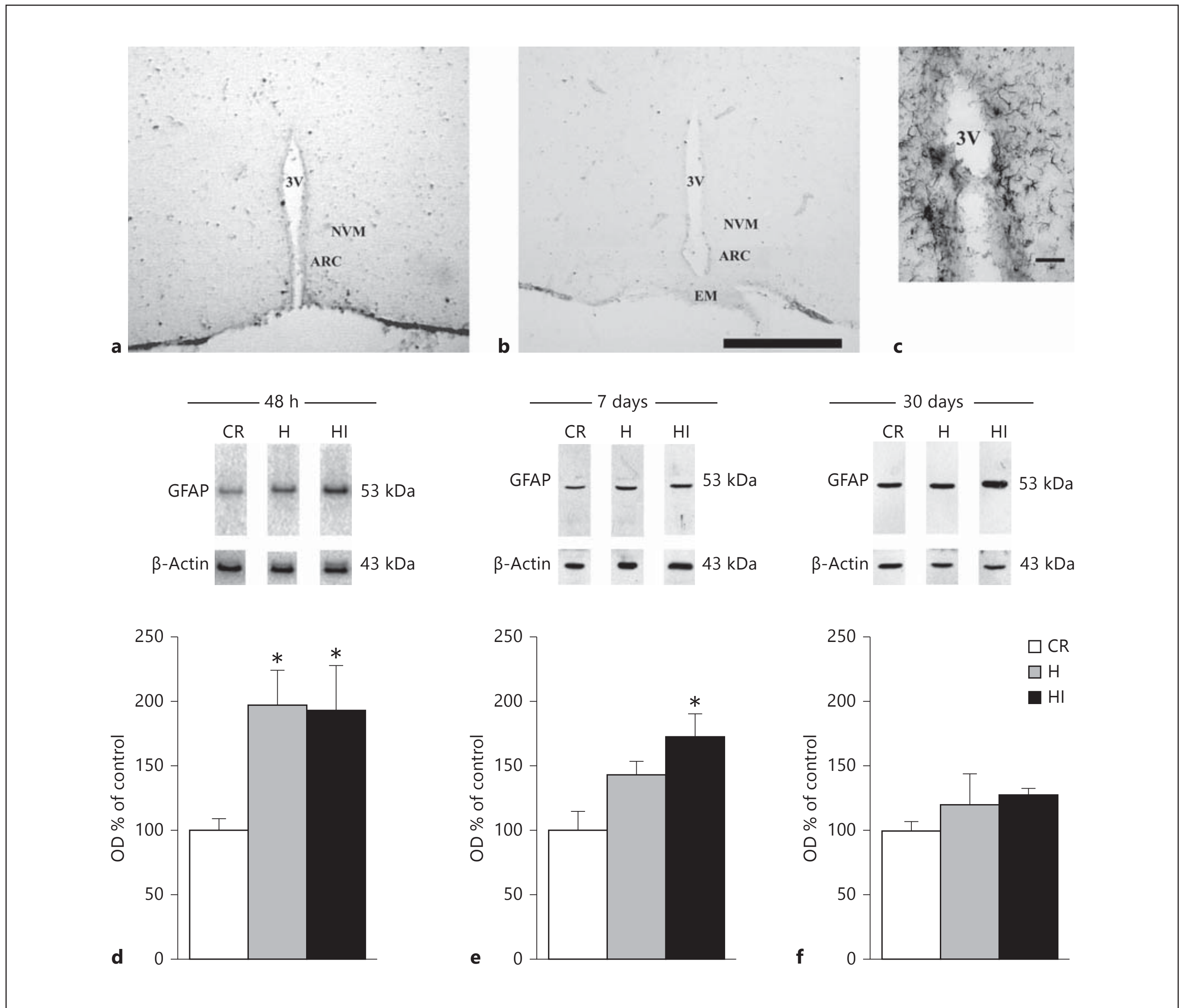

Fig. 1. Astrocyte activation in MBH-PO in CR rats or rats exposed perinatally to H or HI. Astrocyte activation was measured by GFAP immunohistochemistry (**a–c**) and Western blot (**d–f**) at 48 h (Western blot only), 7 and 30 days after injury in the MBH-PO. MBH-PO GFAP+ reactive astrocytes in CR (**a**) and HI (**b**) rats 7 days after injury. Only a few dispersed activated astrocytes were observed in both groups. Some HI animals showed GFAP+ activated astrocytes in the III ventricle (**c**). ARC = Arcuate nucleus; 3V = III ventricle; NVM = ventromedial nucleus; EM = median eminence. Scale bar = 500 μm (**a**, **b**), 25 μm (**c**). GFAP protein levels relative to β-actin at 48 h (**d**), 7 days (**e**) or 30 days (**f**) after injury. * $p < 0.05$ versus CR using one-way ANOVA and τ post hoc test.

18 months when compared with 48 h, and the pattern was similar in the H and HI groups. In contrast, iNOS mRNA was elevated significantly at 7 days in the CR group when compared with the other times studied. Again, this pattern was not altered by H or HI.

IGFBP-3 mRNA showed fluctuations with time in the CR group, with increased values at 7 days (14 days of age) and 8 months. It was significantly increased in HI rats at 48 h, while at 7 days postinjury its mRNA abundance fell in both groups (fig. 3). In contrast, IGFBP-5 mRNA abundance in CR animals remained constant at all times, with the exception of the 30-day group, where the values were significantly diminished. H or HI induced a significant increase in IGFBP-5 mRNA abundance 48 h after

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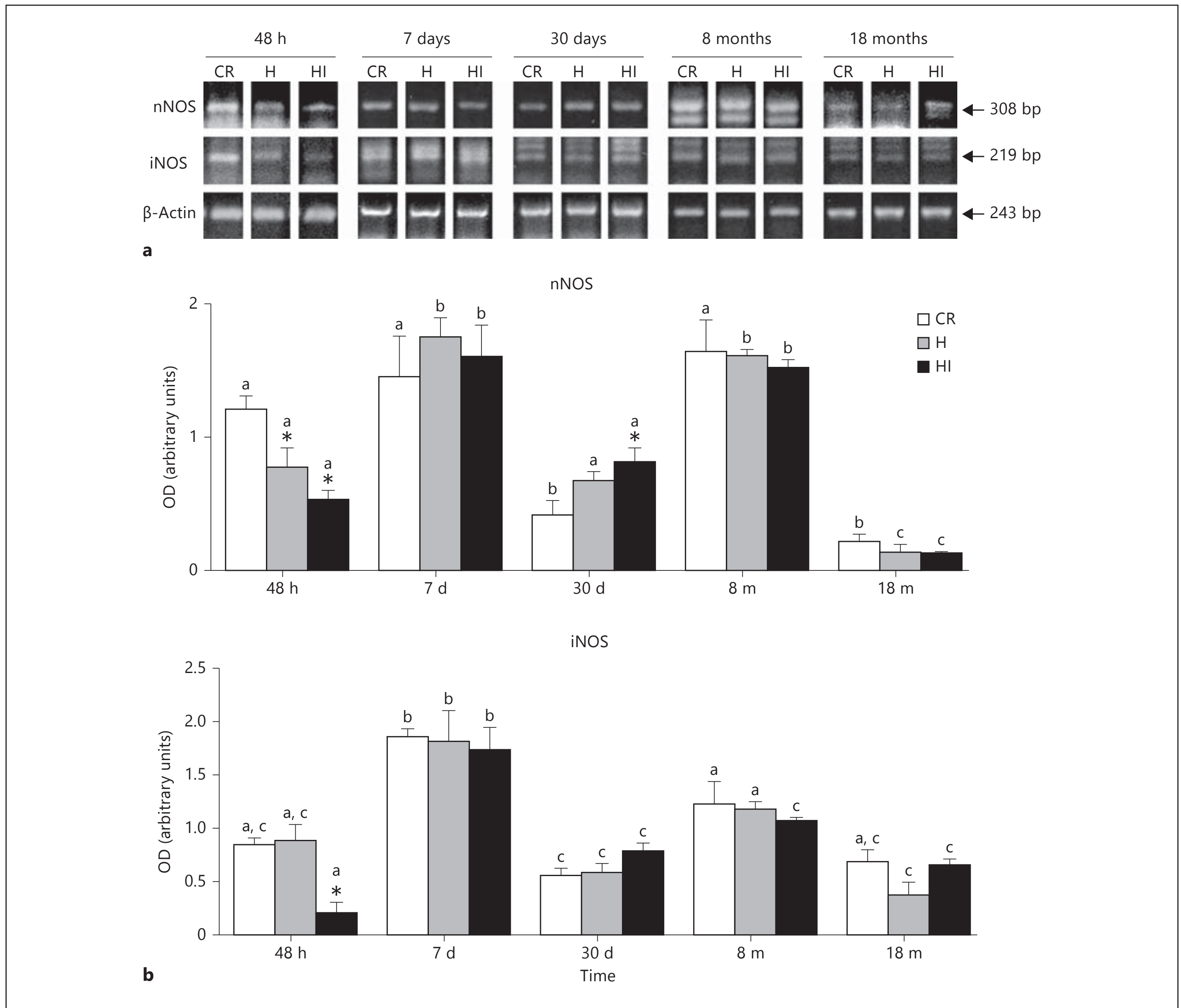

Fig. 2. nNOS and iNOS mRNA levels relative to β-actin in the MBH-PO in CR rats or rats exposed perinatally to H or HI. NOS mRNA levels were measured using RT-PCR on samples obtained at 48 h, 7 and 30 days, and 8 and 18 months after injury. **a** Composite of RT-PCR results showing representative CR and treated rats (H and HI). **b** Semiquantitative analysis of the abundance of NOS isoforms relative to β-actin level. Values represent the mean ± SEM of groups of 6 rats. * $p < 0.05$ compared with the respective CR group using two-way ANOVA and τ post hoc test. Different superscript letters represent significant differences at $p < 0.05$ between different times after the injuries within the same experimental groups.

injury, and the values were also significantly increased in the HI group at 30 days after injury (fig. 3).

IGF-I mRNA abundance showed high values at PND 9 in the CR group and decreased significantly at the later times studied. At 48 h postinjury, the HI group showed a significant decrease, without changes at later times. IGF-IR mRNA abundance increased gradually with age in the CR group, becoming significantly different from the 48-hour group at 8 months. There were no significant differences in the H and HI groups at any time when compared with the respective CR groups, with the exception of the HI group, which was significantly increased 30 days after injury, suggesting an early increase of IGF-IR mRNA abundance in this group (fig. 3).

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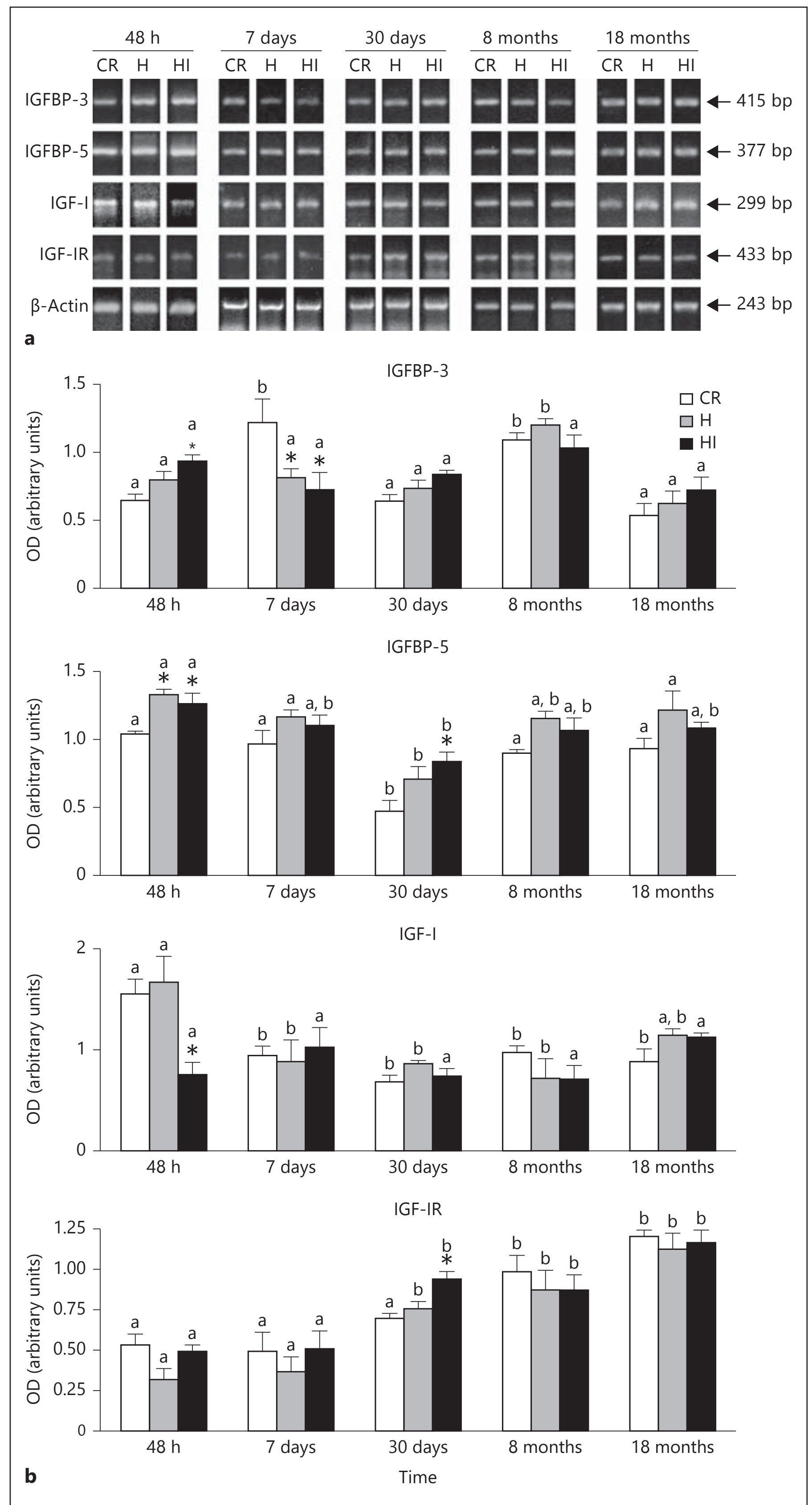

Fig. 3. IGF-I, IGF-IR, IGFBP-3 and IGFBP-5 mRNA levels relative to β-actin in the MBH-PO in CR rats or rats exposed perinatally to H or HI. mRNA levels were measured using RT-PCR on samples obtained at 48 h, 7 and 30 days, and 8 and 18 months after injury. **a** Composite of RT-PCR results showing representative CR and treated rats (H and HI). **b** Semiquantitative analysis of the mRNAs abundance relative to β-actin level. Values represent the mean ± SEM of groups of 6 rats. * $p < 0.05$ compared with the respective CR group using two-way ANOVA and τ post hoc test. Different superscript letters represent significant differences at $p < 0.05$ between different times after the injuries within the same experimental groups.

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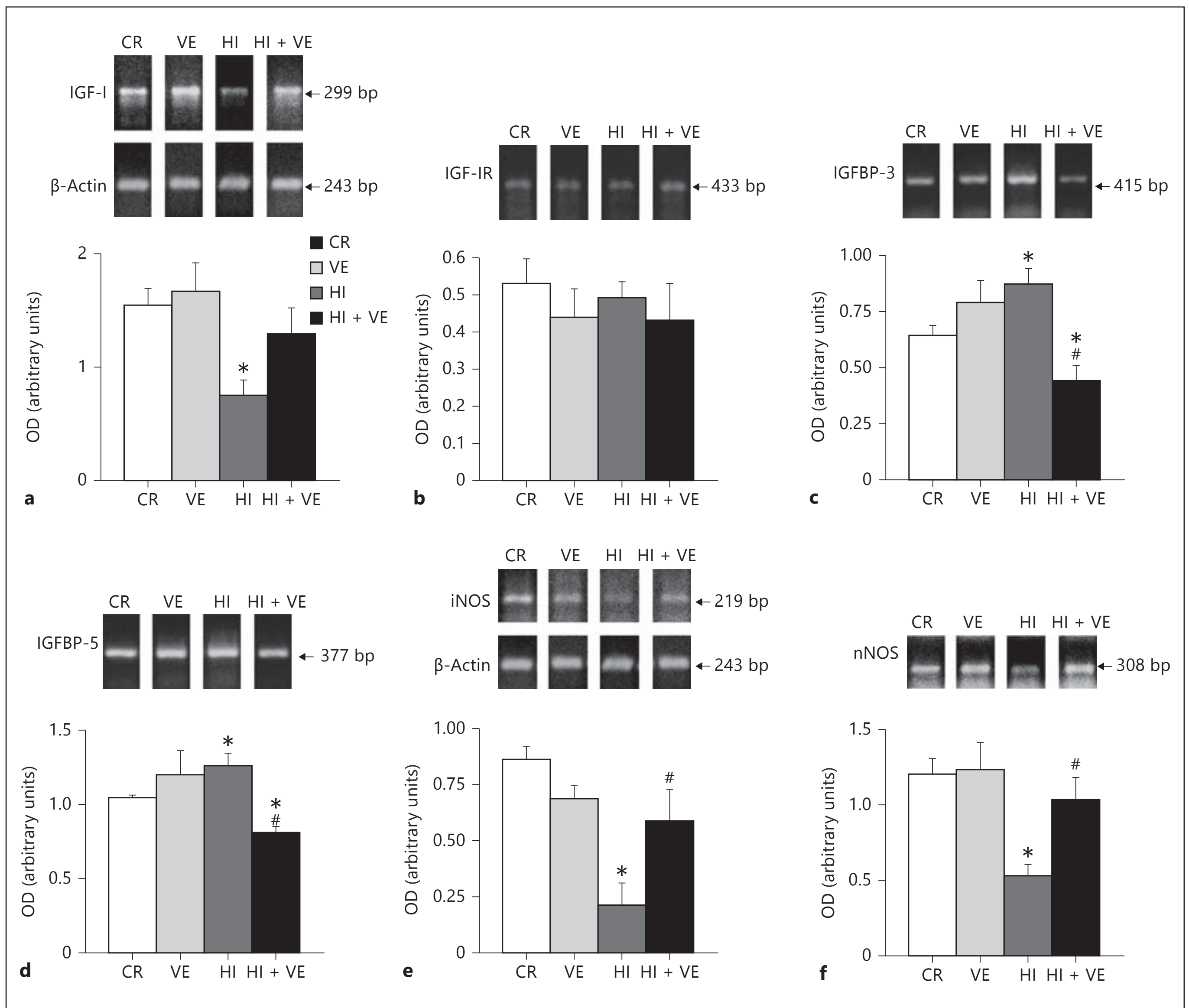

Fig. 4. IGF-I, IGF-IR, IGFBP-3, IGFBP-5, iNOS and nNOS mRNA levels relative to β-actin in the MBH-PO in CR rats or rats exposed perinatally to HI with VE (HI + VE) or without (CR, HI) previous VE treatment. mRNA levels were measured using RT-PCR on samples obtained 48 h after injury showing for each panel a composite of RT-PCR results for representative CR and treated rats, and semiquantitative analysis of the mRNAs abundance relative to β-actin level: IGF-I (**a**); IGF-IR (**b**); IGFBP-3 (**c**); IGFBP-5 (**d**); iNOS (**e**) and nNOS (**f**). Bars represent the mean ± SEM of groups of 6 rats. * $p < 0.05$ versus CR; # $p < 0.05$ versus HI using one-way ANOVA and τ post hoc test.

Effect of VE Treatment on mRNA Content iNOS and nNOS and IGF-Related Proteins in the MBH-PO 48 h after HI

Since we have already shown that the effects of H or HI on reproductive senescence, hormone receptors and inflammation markers were similar [1], we only explored in the HI group whether an antioxidant treatment with VE delivered previous to HI is able to protect from the early effects of the injury. Forty-eight hours after injury there were no significant effects of VE on MBH-PO IGF-IR ($F_{3, 22} = 0.4158$, $p = 0.7436$) and IGFBP-3 and 5, nor on iNOS and nNOS mRNA content, measured by RT-PCR (fig. 4). However, the fall in IGF-I ($F_{3, 22} = 4.425$, $p = 0.0169$) and the increases in IGFBP-3 ($F_{3, 22} = 8.052$, p =

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0.0017) and IGFBP-5 ($F_{3, 22} = 8.002$, $p = 0.0012$) mRNA were prevented by VE treatment (fig. 4a–d). VE treatment also prevented the fall in iNOS ($F_{3, 22} = 8.832$, $p = 0.0007$) and nNOS ($F_{3, 22} = 6.292$, $p = 0.0038$) expression induced by HI (fig. 4e, f).

Effect of VE Treatment on Cycling and Reproductive Senescence on CR and Injured Female Rats

CR rats showed regular 4-day cycles until approximately 7–8 months of age, when they started to show prolonged estrous phases of variable length. As previously described [1], HI rats had a significantly premature onset of prolonged estrus phases, which occurred at 4–5 months of age (fig. 5a), and a marked decrease in the percentage of regular cycling rats from the 4th month of age. VE treatment to CR and HI rats did not significantly modify the regularity of the estrous cycles of the rats or the onset of the prolonged estrus phase in any of the groups (fig. 5a).

However, HI, VE treatment or the combination of both (HI + VE) diminished significantly the preovulatory surge of LH measured at 19.00 h on proestrus (two-way ANOVA: $F_{time, 3, 116} = 40.52$, $p < 0.0001$; $F_{treatments (VE, HI), 3, 116} = 6.202$, $p = 0.0006$; $F_{interaction, 9, 116} = 4.893$, $p < 0.0001$; fig. 5b). FSH values increased significantly in all groups between 12 and 19 h on proestrus, and although there was a tendency for all the VE groups to have higher values than the corresponding untreated groups, and a significant F value for treatments, there were no significant differences at any individual time among any of the groups (two-way ANOVA: $F_{time, 3, 114} = 10.66$, $p < 0.0001$; $F_{treatments (VE, HI), 3, 114} = 4.917$, $p = 0.0030$; $F_{interaction, 9, 114} = 0.5339$, $p = 0.8472$; fig. 5b).

Effect of VE Treatment on mRNA Content of Hormone Receptors in the MBH-PO 48 h after Injury

As previously described [1], the HI episode significantly diminished MBH-PO content of mRNAs of ERβ ($F_{3, 22} = 5.105$, $p = 0.0106$) and ORμ ($F_{3, 22} = 5.742$, $p = 0.0057$), measured by RT-PCR, without affecting ERα ($F_{3, 22} = 2.003$, $p = 0.1496$) or total PR ($F_{3, 22} = 1.015$, $p = 0.4092$) contents. VE treatment significantly diminished ERβ mRNA content without affecting the other receptor mRNAs measured in CR rats (fig. 6), nor were there any significant differences between the HI and HI + VE groups (fig. 6), suggesting that the antioxidant treatment was unable to prevent the effects of HI. Thus, although the VE treatment was effective in attenuating some indicators of HI oxidative damage and the rise of inflammation markers, it was unable to reduce the effects of HI on reproductive senescence.

Discussion

In the present work we have shown that in the MBH-PO, neonatal H or HI episodes induce early changes in the expression of molecules such as NOS and the IGF family that are involved in neuroendocrine regulation of reproductive functions and have been implicated in reproductive aging. Antioxidant treatment with VE was able to prevent these effects in the HI animals, but this treatment was unable to prevent the early reproductive senescence induced by both types of injury, indicating that oxidative damage in the MBH-PO during the neonatal period may not be a major cause of the premature onset of reproductive senescence.

Although the function of reactive astrocytes in neuroprotection or recovery of the central nervous system from an injury is not fully understood, it has been established that activated glia is not necessarily indicative of oxidative stress. It is also known that in this particular model of HI, activated glia plays a protective role forming a scar around the core of the ischemic lesion in order to avoid further damage in the penumbra, preventing a further spreading of the death wave to other regions of the brain [42, 43]. This may be the case of the hypothalamus, which may be protected by these and other mechanisms that safeguard normal neuroendocrine responses and the reproductive function. The lack of presence of macrophages in the MBH-PO confirms that major cell damage has not occurred in this hypothalamic area, and that the detected changes at the molecular level did not cause massive cell death in this region.

NO stimulates hypothalamic GnRH release [44–46]. High levels of ROS and peroxynitrites have been found in the aged brain [47] and are also associated with reproductive senescence [46, 48]. The increase in central nervous system NO production may be due mainly to nNOS activation, although in some hypothalamic regions, such as preoptic area and paraventricular nucleus, increases of iNOS induced by aging have also been found [46, 48]. In contrast, we found decreases in iNOS and nNOS mRNA content in the MBH-PO only 48 h after injury, with no further changes later on. Additionally, we observed that VE was able to prevent the temporary depletion of the synthases mRNA, indicating a positive effect of the antioxidant on NOS expression. We also found early changes in the expression of members of the IGF family, in particular a fall in IGF-I expression in the HI group 48 h after injury, a critical period associated with substantial neurogenesis, synaptogenesis and sex differentiation in the rodent brain [27, 49]. Since IGF-I is a potent neurotrophic

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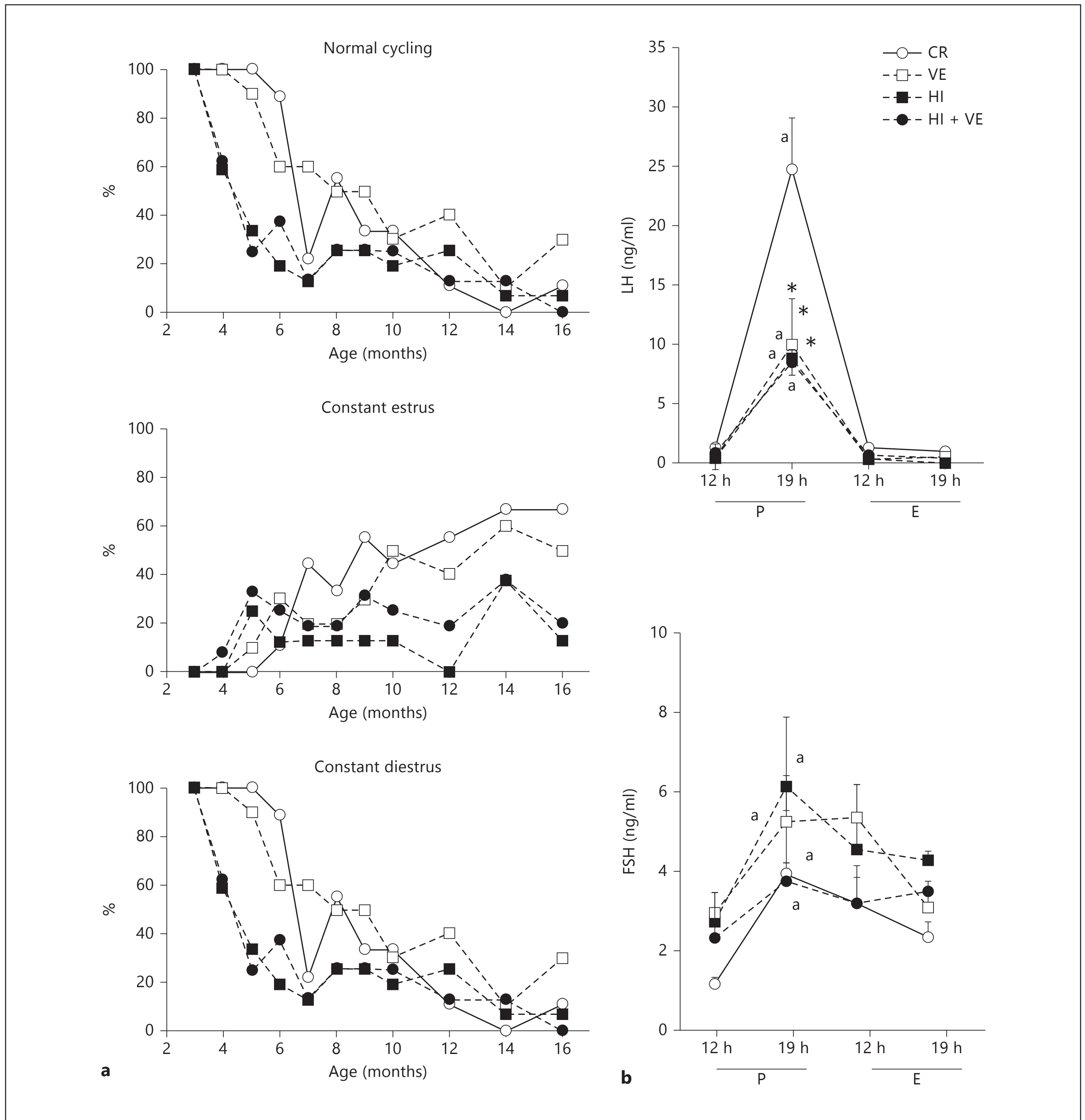

Fig. 5. Effect of perinatal exposure to HI on cyclicity and circulating LH and FSH levels in rats with VE (HI + VE) or without (CR, HI) previous VE treatment. **a** The percentage of rats showing normal cycles, persistent estrus or persistent diestrus between 3 and 16 months of age of groups of 10–15 rats. **b** Circulating LH and FSH levels determined by RIA. To determine the pattern of hormonal secretion during the cycle, rats of each experimental group or CR animals were bled by the tail vein at 12.00 and 19.00 h on proestrus (P) and estrus (E) at 3 months of age. Values represent the means ± SEM of groups of 8–10 rats. * $p < 0.05$ compared with the CR group; [a] $p < 0.05$ compared with proestrus at 12 h using two-way ANOVA and τ post hoc test.

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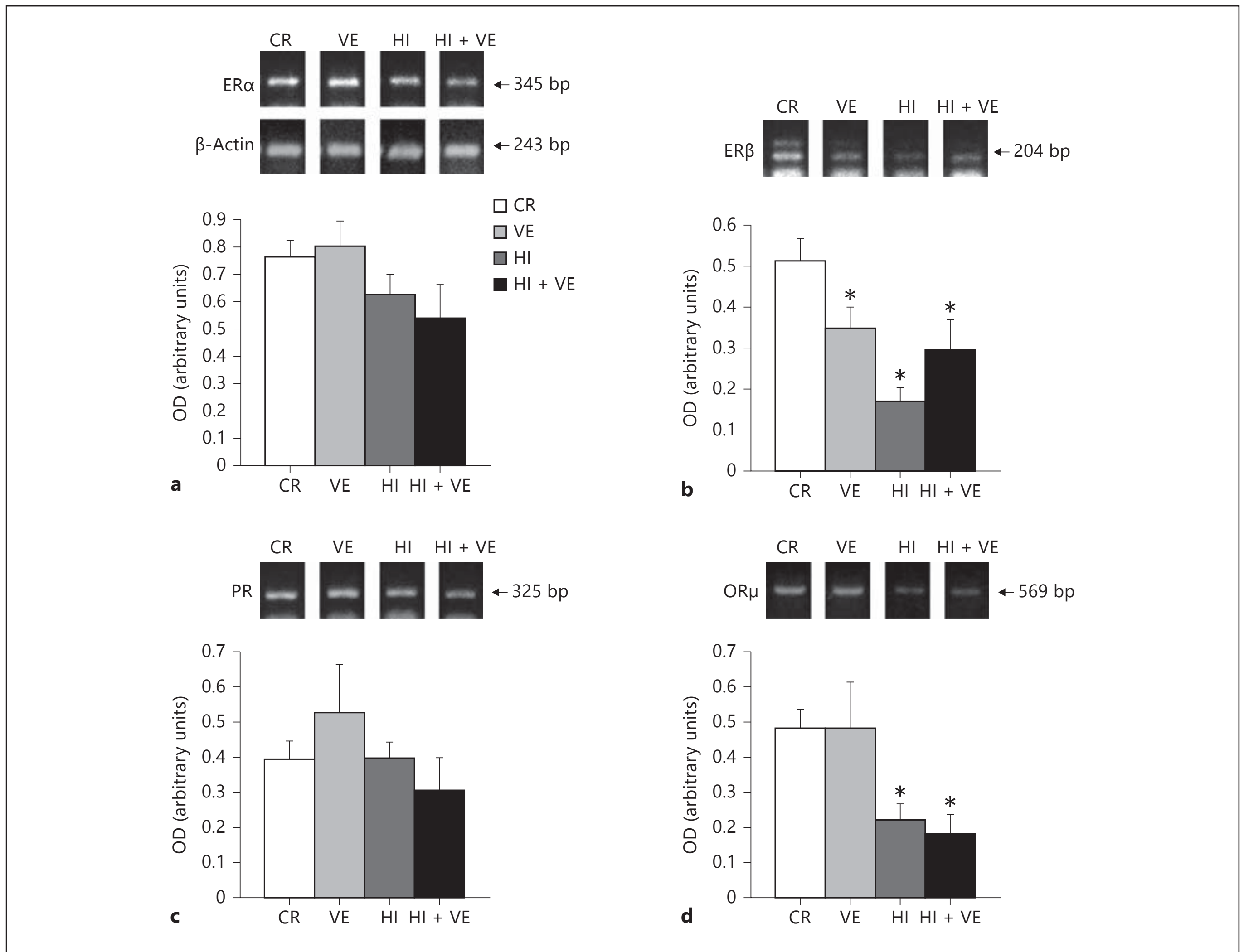

Fig. 6. Hormone receptor mRNA levels in the MBH-PO in CR rats or rats exposed perinatally to HI with VE (HI + VE) or without (CR, HI) previous VE treatment. ERα (**a**), ERβ (**b**), total PR (**c**) and ORμ (**d**) mRNA levels were measured using RT-PCR on samples obtained at 48 h after injury. Each panel shows a composite of RT-PCR results with representative CR and treated rats and the semi-quantitative analysis of the mRNA abundances of PR relative to β-actin level. Values represent the mean ± SEM of groups of 6 rats. * $p < 0.05$ compared with the CR group using one-way ANOVA and Bonferroni post hoc test.

factor during development [50] and a mediator of synaptic plasticity [51], this fall may lead to disruption in the normal development of hypothalamic areas. Between PND 5 and 10, hypothalamic expression of IGF-I is associated with the establishment of the GnRH system, thus, pathological changes in IGF-I production at this time may have long-term reproductive consequences [26, 27]. We also found a significant increase in IGF-IR expression at 30 days postinjury, an important period for reproductive function regulation since it is near puberty and a moment of synaptic consolidation [52]. Thus, these alterations in the IGF system may be related to abnormal responses, since altered brain IGFBP expression has been associated with reproductive dysfunction [53].

Free radical-mediated toxicity has been involved in the reperfusion inflammatory injury produced after HI [8], and increased ROS production has been implicated in normal aging processes [54]. Thus, administration of VE before HI attenuates lipid peroxidation produced during reperfusion [9, 55], and in rats subjected to a VE-deficient diet the exposure to HI increases lipid peroxidation [56].

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However, although VE treatment was able to revert the changes produced by perinatal HI on trophic factors (IGF-I and IGFBPs) and oxidative damage (nNOS and iNOS), most possibly through its action as an ROS scavenger, it was incapable of reverting the premature onset of constant estrus, or the changes observed in hormone receptors such as ERβ and ORμ. A possible explanation for this finding may be that the administration regime used may have been incapable of providing sufficient protection to prevent premature reproductive senescence. Alternatively, VE may not be able to antagonize all the mechanisms activated by HI; for example, ROS damage in the presence of normal concentrations of glutamate (in normoxic or slightly hypoxic situations) can be attenuated by free radical scavengers such as VE [57], but more serious damage may involve the activation of additional cell survival strategies.

Interactions between estrogens and the IGF-I system have been well documented in brain areas related to reproduction, among them the hypothalamus. Thus, estrogens can regulate the expression at mRNA and protein levels of IGF-I, IGFBPs and IGF-IR; inversely, IGF-I can modulate the expression and function of ERs [27]. In particular, the interaction between estrogens and IGF-I participates in the plastic changes in synaptic connections and glial-neuronal contacts observed during puberty and through the estrous cycle [27]. HI produced early changes in the expression of ERβ and various members of the IGF family, which may be related to premature reproductive senescence; however, although VE treatment was able to revert in part the changes observed in IGF-I and completely the changes observed in IGFBP-3 and 5, it did not modify the fall on ERβ expression produced by HI, and lowered ERβ expression by itself, indicating that HI-induced premature reproductive senescence may not be related to early changes in ER expression, and that the fall in ERβ may not be mediated by the IGF-I system. During development, the hormone-receptor interactions produce neuronal imprinting that influences the future interaction between hormones and their receptors and, therefore, the neuronal responses. Abnormal interactions with molecules that can bind to the receptors with high affinity may result in abnormal imprinting, which may alter the binding capacity of the receptors and their responses [58, 59]. VE has been shown to have this type of interaction with steroid hormone receptors [60, 61], which may be the cause of the decreased ERβ expression.

The neonatal VE treatment resulted in reduced preovulatory LH release at 19.00 h on proestrus in adult rats (fig. 5) and in reduced ERβ expression in neonatal rats (fig. 6). We cannot exclude that the neonatal VE treatment may have produced slight alterations in the timing of the preovulatory peaks, resulting in reduced levels at 19.00 h, but in an adequate preovulatory peak. VE has been shown to bind selectively to ERβ in mammary cancer cell lines and act as a very weak agonist [62]. VE can also stimulate hypothalamic GnRH release [63], is needed for normal gonadal function and gonadotropin secretion in adult rodents [64, 65], and can protect from the reproductive endocrine toxicity produced by some environmental contaminants [66, 67].

ERβ and ERβ-related proteins colocalize with GnRH in hypothalamic neurons in mice and rats [68], and have been shown to mediate the firing, cAMP production, galanin (a potent GnRH releaser) expression and GnRH release of these neurons [69–71]. Thus, any effect of VE on neonatal expression of ERβ, during a moment of maturation of these structures, may well affect the future function of these neurons and the regulation of the preovulatory LH surges. However, VE treatment did not affect cyclicity and fertility, indicating that the amount of LH released during the preovulatory peak was sufficient to ensure ovulation and that the early reduction in ERβ expression is not sufficient to modify fertility or the span of the reproductive stage in female rats.

In conclusion, perinatal H or HI induces an altered expression of factors, such as iNOS and nNOS and members of the IGF-I family that play roles in the regulation of estrous cycle periodicity and GnRH secretion. These alterations are most probably mediated by ROS produced during and after the hypoxic episode, since the antioxidant effect of the treatment with VE prevented the changes on mRNA expression of IGF-I, IGFBP-3 and IGFBP-5, iNOS and nNOS induced by HI. However, the reproductive alterations and the changes in steroid hormone and opioid receptors produced by HI were not prevented by treatment with the antioxidant. On the other hand, the changes in the expression of ERβ and ORμ that were not prevented by VE may be most likely associated with the premature onset of reproductive cycle irregularities.

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References

1 Ezquer M, Valdez SR, Seltzer AM, Jahn GA: Advance of reproductive senescence and changes in the early expression of oestrogen, progesterone and μ-opioid receptors induced by neonatal hypoxia in the female rat. Brain Res 2008;1214:73–83.

2 Mills RH, Romeo HE, Lu JK, Micevych PE: Site-specific decrease of progesterone receptor mRNA in the hypothalamus of middle-aged persistently estrus rats. Brain Res 2002;955:200–206.

3 Chakraborty TR, Gore AC: Aging-related changes in ovarian hormones, their receptors, and neuroendocrine function. Exp Biol Med 2004;229:977–987.

4 Brann DW, Mahesh VB: The aging reproductive neuroendocrine axis. Steroids 2005;70:273–283.

5 Fukui K, Onodera K, Shinkai T, Suzuki S, Urano S: Impairment of learning and memory in rats caused by oxidative stress and aging, and changes in antioxidative defense systems. Ann NY Acad Sci 2001;928:168–175.

6 Kobayashi N, Machida T, Takahashi T, Takatsu H, Shinkai T, Abe K, Urano S: Elevation by oxidative stress and aging of hypothalamic-pituitary-adrenal activity in rats and its prevention by vitamin E. J Clin Biochem Nutr 2009;45:207–213.

7 Takatsu H, Owada K, Abe K, Nakano M, Urano S: Effect of vitamin E on learning and memory deficit in aged rats. J Nutr Sci Vitaminol 2009;55:389–393.

8 Romero-Ramos M, Venero JL, Santiago M, Rodriguez-Gomez JA, Vizuete ML, Cano J, Machado A: Decreased messenger RNA expression of key markers of the nigrostriatal dopaminergic system following vitamin E deficiency in the rat. Neuroscience 2000;101:1029–1036.

9 Aabdallah DM, Eid NI: Possible neuroprotective effects of lecithin and α-tocopherol alone or in combination against ischemia/reperfusion insult in rat brain. J Biochem Mol Toxicol 2004;18:273–278.

10 Hara H, Kato H, Kogure K: Protective effect of α-tocopherol on ischemic neuronal damage in the gerbil hippocampus. Brain Res 1990;510:335–338.

11 Hara H, Kogure K, Kato H, Ozaki A, Sukamoto T: Amelioration of brain damage after focal ischemia in the rat by a novel inhibitor of lipid peroxidation. Eur J Pharmacol 1991;197:75–82.

12 Hung AJ, Stanbury MG, Shanabrough M, Horvath TL, Garcia-Segura LM: Estrogen, synaptic plasticity and hypothalamic reproductive aging. Exp Gerontol 2003;38:53–59.

13 Lu JKH, Matt DW, LaPolt PS: Modulatory effects of estrogen and progestin on female reproductive aging; in Naftolin F, DeCherney AH, Gutmann JN, Sarell PM (eds): Ovarian Secretions and Cardiovascular and Neurological Function. Philadelphia, Raven Press, 1990, vol xx, pp 297–306.

14 Perez J, Luquin S, Naftolin F, García-Segura LM: The role of estradiol and progesterone in phased synaptic remodelling of the rat arcuate nucleus. Brain Res 1993;608:38–44.

15 Godbout JP, Berg BM, Kelley KW, Johnson RW: α-Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6 secretion in primary murine microglia and in brain. J Neuroimmunol 2004;149:101–109.

16 Brigelius-Flohe R, Kelly FJ, Salonen JT, Neuzil J, Zingg JM, Azzi A: The European perspective on vitamin E: current knowledge and future research. Am J Clin Nutr 2002;76:703–716.

17 Erin AN, Spirin MM, Tabidze LV, Kagan VE: Formation of α-tocopherol complexes with fatty acids: a hypothetical mechanism of stabilization of biomembranes by vitamin E. Biochim Biophys Acta 1984;774:96–102.

18 Sokol RJ: Vitamin E and neurologic deficits. Adv Pediatr 1990;37:119–148.

19 Kohsaka A, Watanobe H, Kakizaki Y, Suda T: A comparative study of the effects of nitric oxide and carbon monoxide on the in vivo release of gonadotropin-releasing hormone and neuropeptide Y from rat hypothalamus during the estradiol-induced luteinizing hormone surge: estimation by push-pull perfusion. Neuroendocrinology 1999;69:245–253.

20 Costa A, Trainer P, Besser M, Grossman A: Nitric oxide modulates the release of corticotropin-releasing hormone from the rat hypothalamus in vitro. Brain Res 1993;605:187–192.

21 Ceccatelli S, Hulting AL, Zhang X, Gustafsson L, Villar M, Hokfelt T: Nitric oxide synthase in the rat anterior pituitary gland and the role of nitric oxide in regulation of luteinizing hormone secretion. Proc Natl Acad Sci USA 1993;90:11292–11296.

22 Duvilanski BH, Zambruno C, Seilicovich A, Pisera D, Lasaga M, Diaz MC, Belova N, Rettori V, McCann SM: Role of nitric oxide in control of prolactin release by the adenohypophysis. Proc Natl Acad Sci USA 1995;92:170–174.

23 Okamura H, Yokosuka M, Hayashi S: Estrogenic induction of NADPH-diaphorase activity in the preoptic neurons containing estrogen receptor immunoreactivity in the female rat. J Neuroendocrinol 1994;6:597–601.

24 Ceccatelli S, Eriksson M: The effect of lactation on nitric oxide synthase gene expression. Brain Res 1993;625:177–179.

25 Hatakeyama S, Kawai Y, Ueyama T, Senba E: Nitric oxide synthase-containing magnocellular neurons of the rat hypothalamus synthesize oxytocin and vasopressin and express *Fos* following stress stimuli. J Chem Neuroanat 1996;11:243–256.

26 Daftary SS, Gore AC: Developmental changes in hypothalamic insulin-like growth factor-1: relationship to gonadotropin-releasing hormone neurons. Endocrinology 2003;144:2034–2045.

27 Daftary SS, Gore AC: IGF-1 in the brain as a regulator of reproductive neuroendocrine function. Exp Biol Med 2005;230:292–306.

28 Carro E, Trejo JL, Busiguina S, Torres-Aleman I: Circulating insulin-like growth factor I mediates the protective effects of physical exercise against brain insults of different etiology and anatomy. J Neurosci 2001;21:5678–5684.

29 Cheng CM, Reinhardt RR, Lee WH, Joncas G, Patel SC, Bondy CA: Insulin-like growth factor 1 regulates developing brain glucose metabolism. Proc Natl Acad Sci USA 2000;97:10236–10241.

30 Carro E, Nunez A, Busiguina S, Torres-Aleman I: Circulating insulin-like growth factor I mediates effects of exercise on the brain. J Neurosci 2000;20:2926–2933.

31 Anderson MF, Aberg MA, Nilsson M, Eriksson PS: Insulin-like growth factor-I and neurogenesis in the adult mammalian brain. Brain Res Dev Brain Res 2002;134:115–122.

32 Thornton PL, Sonntag W: Growth hormone, insulin-like growth factor 1 and the aging brain; in Hof PR, Mobbs CV (eds): Functional Neurobiology of Aging. San Diego, Academic Press, 2001, pp 907–928.

33 Ezquer ME, Valdez SR, Seltzer AM: Inflammatory responses of the substantia nigra after acute hypoxia in neonatal rats. Exp Neurol 2006;197:391–398.

34 Ezquer M, Seltzer AM: c-fos and tyrosine hydroxylase expression after an excitotoxic lesion on the nigrostriatal system: a study on the effects of hypoxia used as a preconditioning stimulus. Exp Neurol 2003;183:523–531.

35 Vannucci RC: Experimental models of perinatal hypoxic-ischemic brain damage. APMIS Suppl 1993;40:89–95.

36 Trescher WH, Ishiwa S, Johnston MV: Brief post-hypoxic-ischemic hypothermia markedly delays neonatal brain injury. Brain Dev 1997;19:326–338.

37 Paxinos G, Watson C: Atlas: The Rat Brain in Stereotaxic Coordinates, ed 7. London, Elsevier, 2007.

38 Valdez SR, Patterson SI, Ezquer ME, Torrecilla M, Lama MC, Seltzer AM: Acute sublethal global hypoxia induces transient increase of GAP-43 immunoreactivity in the striatum of neonatal rats. Synapse 2007;61:124–137.

39 Middeldorp J, Hol EM: GFAP in health and disease. Prog Neurobiol 2011;93:421–443.

40 Wu D, Miyamoto O, Shibuya S, Okada M, Igawa H, Janjua NA, Norimatsu H, Itano T: Different expression of macrophages and microglia in rat spinal cord contusion injury model at morphological and regional levels. Acta Med Okayama 2005;59:121–127.

41 Damoiseaux JG, Dopp EA, Calame W, Chao D, MacPherson GG, Dijkstra CD: Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. Immunology 1994;83:140–147.

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42 Shrivastava K, Chertoff M, Llovera G, Recasens M, Acarin L: Short and long-term analysis and comparison of neurodegeneration and inflammatory cell response in the ipsilateral and contralateral hemisphere of the neonatal mouse brain after hypoxia/ischemia. Neurol Res Int 2012;2012:781512.
43 Kawano H, Kimura-Kuroda J, Komuta Y, Yoshioka N, Li HP, Kawamura K, Li Y, Raisman G: Role of the lesion scar in the response to damage and repair of the central nervous system. Cell Tissue Res 2012;349:169–180.
44 Lopez FJ, Moretto M, Merchenthaler I, Negro-Vilar A: Nitric oxide is involved in the genesis of pulsatile LHRH secretion from immortalized LHRH neurons. J Neuroendocrinol 1997;9:647–654.
45 Chen H, Huhtaniemi I, Zirkin BR: Depletion and repopulation of Leydig cells in the testes of aging brown Norway rats. Endocrinology 1996;137:3447–3452.
46 Ferrini M, Wang C, Swerdloff RS, Sinha Hikim AP, Rajfer J, Gonzalez-Cadavid NF: Aging-related increased expression of inducible nitric oxide synthase and cytotoxicity markers in rat hypothalamic regions associated with male reproductive function. Neuroendocrinology 2001;74:1–11.
47 Sastre J, Pallardo FV, Vina J: Mitochondrial oxidative stress plays a key role in aging and apoptosis. IUBMB Life 2000;49:427–435.
48 Vernet D, Bonavera JJ, Swerdloff RS, Gonzalez-Cadavid NF, Wang C: Spontaneous expression of inducible nitric oxide synthase in the hypothalamus and other brain regions of aging rats. Endocrinology 1998;139:3254–3261.
49 Pang SF, Tang F: Sex differences in the serum concentrations of testosterone in mice and hamsters during their critical periods of neural sexual differentiation. J Endocrinol 1984; 100:7–11.
50 De Pablo F, De La Rosa EJ: The developing CNS: a scenario for the actions of proinsulin, insulin, and insulin-like growth factors. Trends Neurosci 1995;18:143–150.
51 Torres-Aleman I: Insulin-like growth factors as mediators of functional plasticity in the adult brain. Horm Metab Res 1999;31:114–119.
52 Daftary SS, Gore AC: The hypothalamic insulin-like growth factor-1 receptor and its relationship to gonadotropin-releasing hormones neurones during postnatal development. J Neuroendocrinol 2004;16:160–169.
53 Froment P, Staub C, Hembert S, Pisselet C, Magistrini M, Delaleu B, Seurin D, Levine JE, Johnson L, Binoux M, Monget P: Reproductive abnormalities in human insulin-like growth factor-binding protein-1 transgenic male mice. Endocrinology 2004;145:2080–2091.
54 Southorn PA, Powis G: Free radicals in medicine. II. Involvement in human disease. Mayo Clin Proc 1988;63:390–408.
55 Villalobos MA, De la Cruz JP, Carrasco T, Smith-Agreda JM, Sanchez de la Cuesta F: Effects of alpha-tocopherol on lipid peroxidation and mitochondrial reduction of tetraphenyl tetrazolium in the rat brain. Brain Res Bull 1994;33:313–318.
56 Yoshida S, Busto R, Santiso M, Ginsberg MD: Brain lipid peroxidation induced by postischemic reoxygenation in vitro: effect of vitamin E. J Cereb Blood Flow Metab 1984;4:466–469.
57 Meydani M, Lipman RD, Han SN, Wu D, Beharka A, Martin KR, Bronson R, Cao G, Smith D, Meydani SN: The effect of long-term dietary supplementation with antioxidants. Ann NY Acad Sci 1998;854:352–360.
58 Csaba G, Inczefi-Gonda A: Neonatal vitamin E treatment induces long term glucocorticoid receptor changes: an unusual hormonal imprinting effect. Life Sci 1998;63:101–105.
59 Csaba G, Inczefi-Gonda A: Effect of vitamin D_3 treatment in the neonatal or adolescent age (hormonal imprinting) on the thymic glucocorticoid receptor of the adult male rat. Horm Res 1999;51:280–283.
60 Chamras H, Barsky SH, Ardashian A, Navasartian D, Heber D, Glaspy JA: Novel interactions of vitamin E and estrogen in breast cancer. Nutr Cancer 2005;52:43–48.
61 Azzi A, Ricciarelli R, Zingg JM: Non-antioxidant molecular functions of α-tocopherol (vitamin E). FEBS Lett 2002;519:8–10.
62 Comitato R, Nesaretnam K, Leoni G, Ambra R, Canali R, Bolli A, Marino M, Virgili FA: Novel mechanism of natural vitamin E tocotrienol activity: involvement of ER beta signal transduction. Am J Physiol Endocrinol Metab 2009;297:E427–E437.
63 Karanth S, Yu WH, Mastronardi CA, McCann SM: Vitamin E stimulates luteinizing hormone-releasing hormone and ascorbic acid release from medial basal hypothalami of adult male rats. Exp Biol Med 2003;228:779–785.
64 Akazawa N, Mikami S, Kimura S: Effects of vitamin E deficiency on the hormone secretion of the pituitary-gonadal axis of the rat. Tohoku J Exp Med 1987;152:221–229.
65 Cooper DR, Kling OR, Carpenter MP: Effect of vitamin E deficiency on serum concentrations of follicle-stimulating hormone and testosterone during testicular maturation and degeneration. Endocrinology 1987;120:83–90.
66 Selvakumar K, Sheerin Banu L, Krishnamoorthy G, Venkataraman P, Elumalai P, Arunakaran J: Differential expression of androgen and estrogen receptors in PCB (Aroclor 1254)-exposed rat ventral prostate: impact of alpha-tocopherol. Exp Toxicol Pathol 2011; 63:105–112.
67 Yin HP, Xu JP, Zhou XQ, Wang Y: Effects of vitamin E on reproductive hormones and testis structure in chronic dioxin-treated mice. Toxicol Ind Health 2012;28:152–161.
68 Herbison AE, Pape JR: New evidence for estrogen receptors in gonadotropin-releasing hormone neurons. Front Neuroendocrinol 2001;22:292–308.
69 Hu L, Gustofson RL, Feng H, Leung PK, Mores N, Krsmanovic LZ, Catt KJ: Converse regulatory functions of estrogen receptor-α and -β subtypes expressed in hypothalamic gonadotropin-releasing hormone neurons. Mol Endocrinol 2008;22: 2250–2259.
70 Merchenthaler I, Hoffman GE, Lane MV: Estrogen and estrogen receptor-β (ERβ)-selective ligands induce galanin expression within gonadotropin hormone-releasing hormone-immunoreactive neurons in the female rat brain. Endocrinology 2005;146:2760–2765.
71 Wolfe A, Wu S: Estrogen receptor-β in the gonadotropin-releasing hormone neuron. Semin Reprod Med 2012;30:23–31.

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