Neurochemistry International 58 (2011) 273–280

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Partial neuroprotection by 17-β-estradiol in neonatal gamma-irradiated rat cerebellum

Maria A. Zorrilla Zubilete [a,*], Laura R. Guelman [a], Damian G. Maur [a], Lucila G. Caceres [a], Hugo Rios [b], Luis M. Zieher [a], Ana M. Genaro [a]

[a] 1a Cátedra de Farmacología-Centro de Investigaciones Farmacológicas y Botánicas Facultad de Medicina, Universidad de Buenos Aires-CONICET, Paraguay 2155, Piso 15, 1121 Buenos Aires, Argentina
[b] Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis", Facultad de Medicina, Universidad de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 25 March 2010
Received in revised form 7 November 2010
Accepted 25 November 2010
Available online 14 December 2010

Keywords:
γ-Irradiation
Neuroprotection
Estrogen
Nitric oxide synthase
Protein kinase C

ABSTRACT

Acute and long-term complications can occur in patients receiving radiation therapy. It has been suggested that cytoprotection might decrease the incidence and severity of therapy-related toxicity in these patients. Developing cerebellum is highly radiosensitive and for that reason it is a useful structure to test potential neuroprotective substances to prevent radiation induced abnormalities.

Recent studies have shown that estrogen can rapidly modulate intracellular signalling pathways involved in cell survival. Thus, it has been demonstrated that estrogens mediate neuroprotection by promoting growth, cell survival and by preventing axonal pruning.

The aim of this work was to evaluate the effect of the treatment with 17-β-estradiol on the motor, structural and biochemical changes induced by neonatal ionizing radiation exposure, and to investigate the participation of nitric oxide and protein kinase C, two important intracellular messengers involved in neuronal activity. Our results show that perinatal chronic 17-β-estradiol treatment partially protects against radiation-induced cerebellar disorganization and motor abnormalities. PKC and NOS activities could be implicated in its neuroprotective mechanisms. These data provide new evidence about the mechanisms underlying estrogen neuroprotection, which could have therapeutic relevance for patients treated with radiotherapy.

1. Introduction

Acute and long-term complications can occur in patients receiving radiation therapy. It has been suggested that cytoprotection might decrease the incidence and severity of therapy-related toxicity in these patients. Previous findings in our laboratory showed that neonatal ionizing radiation induces permanent abnormalities in cerebellar cortex cytoarchitecture (granule cell loss and disarrangement of Purkinje cell monolayer), with increased levels of noradrenaline, increased activity of protein kinase C (PKC) PKC and diminished activity of nitric oxide synthase (NOS) in the cerebellum, as well as an impairment in motor gait (ataxic gait) (Dopico et al., 1989; Zorrilla Zubilete et al., 2005). These results have demonstrated that during development, the cerebellum is highly radiosensitive and for this reason it is a useful tool to test potential neuroprotective substances to prevent radiation-induced abnormalities.

Epidemiological studies have shown that estrogen therapy soon after the menopause is associated with numerous beneficial health effects, including decreased risk of neurodegenerative diseases (Zandi et al., 2002) and increased cognitive performance (Henderson et al., 1994). Experimental data in animal models have provided exhaustive evidence of the neuroprotective properties of 17-β-estradiol (E2). This hormone increases neuronal survival after different forms of brain injury in vivo and protects neuronal cultures from serum or growth factor deprivation, anoxia, excitotoxicity or oxidative damage (reviewed in Green and Simpkins, 2000; Lee and Mc Ewen, 2001).

This neuroprotective effect is not due to a single phenomenon but rather encompasses a spectrum of independent proccesses. Estrogens directly promote cell survival, synaptic plasticity and prevent axonal and dendritic pruning. Estrogens can also prevent neuronal malfunction by modulating the levels of neurotransmitters, its receptors and second messengers. Thus, the protective effects of estrogens appears to be multifacetic (reviewed in Garcia-Segura et al., 2001).

* Corresponding author at: Paraguay 2155, Piso 15°, 1a Cátedra de Farmacología, Facultad de Medicina, U.B.A., Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 4508 3733; fax: +54 11 4508 3733.
E-mail address: mariazorrillaz@gmail.com (M.A. Zorrilla Zubilete).

0197-0186/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2010.11.020

Recent studies suggest that physiological levels of estrogen can also rapidly modulate diverse intracellular signalling pathways (reviewed in Kelly and Levin, 2001), several of which have been shown to regulate cell viability (Dudek et al., 1997; Walton et al., 1999). One important mediator of intracellular signalling is PKC, a family of 12 serine/threonine kinases. PKC has been found to regulate several cellular events such as cell cycle progression, neuronal signalling and apoptosis (reviewed in Mellor and Parker, 1998). Activation of PKC modulates cell viability pathways, resulting in protection of non-neuronal (Liu et al., 2001) and neuronal cells (Xie et al., 2000; Maher, 2001). However, activation of PKC can also contribute to cell death (Datta et al., 1997). It has been shown that estrogen-induced neuroprotection of primary cerebrocortical neurons from beta-amyloid peptide toxicity depends on activation of conventional PKC (Cordey et al., 2003; Cordey and Pike, 2006).

Nitric oxide (NO), probably the smallest and most versatile bioactive molecule identified, plays an important role in the central nervous system (Dawson and Snyder, 1994) being involved in both physiological and pathological processes (Dawson and Dawson, 1996; Yun et al., 1997). In addition, it plays an important role in the control of neural activity by diffusing into neurons, and participates in learning and memory processes (Chen et al., 1997). It has been demonstrated that NO has a significant function in long term potentiation (LTP) in the hippocampus (Hawkins et al., 1998) and long term depression (LTD) in the cerebellum (Lev-Ram et al., 1995). NO is synthesized from L-arginine by the NOS. At least three distinct isoforms of NOS have been cloned and located: endothelial NOS (eNOS) and neuronal NOS (nNOS), having calcium-dependent activity and being constitutively expressed in endothelial cells and in neurons, respectively, and a calcium independent subtype known as inducible NOS (iNOS) (Bredt and Snyder, 1990). There is evidence that NOS can be phosphorylated by different kinases, including PKC. PKC phosphorylation has been shown to either stimulate (Nakane et al., 1991) or inhibit (Bredt et al., 1992; Dawson and Snyder, 1994) NOS activity in vitro, whereas in transfected cells it clearly inhibits the catalytic activity of the enzyme.

The aim of the present work was to examine the potential protective properties of E2 on the motor and neurochemical alterations in rat's cerebellum induced by neonatal gamma-irradiation using a previously described neuroprotection assessment protocol (Guelman et al., 2001). Specifically, the participation of PKC and NO in the neuroprotective effects of E2 was also studied. On the other hand, it has been shown that neuroprotective agents are effective in counteracting ROS toxicity after different types of neuronal injury (Ishikawa et al., 1999; Almli et al., 2001; Eshhar et al., 1995). Previously, using an *in vitro* radiation model, we demonstrated that an iron chelator (deferoxamine) and a ROS scavenger (amifostine) were able to prevent cell death (Guelman et al., 2004, 2005). Therefore, the potential antioxidant mechanism for E2 will be evaluated in the present paper.

2. Experimental procedures

2.1. Materials

Dowex AG 50WX-8 was purchased from Bio Rad and DE-52 from Whatman & Co. [^{32}P]-γ ATP (Specific Activity 6000 Ci/mmol) was provided by New England Nuclear. All other reagents were provided by Sigma Chemical Co. St. Louis, MO, USA.

2.2. Animals

Newborn littermate Wistar rats from both sexes (10 pups per litter) were maintained with their dams until weaning and allowed free access to food and water. The light–dark cycle was 12:12 (h) and room temperature (RT) was held constant at 25 ± 2 °C. Animals were separated into different experimental groups, following random mixing across multiple dams: control (C), control + 17-β-estradiol (E2), gamma-irradiated (IR) and gamma-irradiated +17-β-estradiol (IR + E2). All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996 and approved by the Institutional Commitee for the care and use of laboratory animals (CICUAL), School of Medicine, University of Buenos Aires.

2.3. Irradiation procedure

Wistar rats were exposed to γ-rays, using a ^{60}Co source with a total single dose of 5 Gy applied to the head. The dose rate of 1 Gy/min was delivered by a cobalt beam (Theratron 80). The exposure time was fixed by previous dosimetry. Littermates from IR and IR + E2 experimental groups were exposed for 5 min, between 36 and 48 h after birth, focusing the radiation field just over the pup's head, allowing the exposure only in the rat's cephalic end. Rats from the control group were handled similarly to the their respective littermates from the γ-irradiated groups, but without being exposed to radiation (SHAM group).

2.4. Pharmacological treatment

Acute and chronic treatment with E2 were performed. For this purpose, 1 μg of 17-β-estradiol per g of body weight was dissolved in 100 μl of saline. For acute treatment, only one subcutaneous (*sc*) injection was administered 30 min before irradiation. For chronic treatment, five doses were administered (total dose = 5 μg/g) to neonatal pups, the first beginning 30 min before exposure, another dose 30 min after the irradiation exposure (or sham exposure), following by one dose per day during three days to E2 and E2 + IR groups. Pups from groups C and IR were injected with equal volumes of isotonic saline (see schedule in Fig. 1).

2.5. Histological procedure

2.5.1. Calbindin D28K immunohistochemistry

Perfusion fixation of cerebella was performed according to a modification of the original method (Gonzalez-Aguilar and De Robertis, 1963). Animals were perfused intracardiacally with a solution of 4% paraformaldehyde (w/v) in 0.1 M phosphate buffer, pH 7.3–7.5. After dissecting out, the cerebella were placed in the same fixative solution for 6–8 h at 4–5 °C. Cerebella were sliced coronally with an Oxford vibratome and sections (40–50 μm thick) were stored at 5 °C in 0.85% NaCl 0.05 M, Tris–HCl pH 7.5 (TS) or dehydrated in ethanol, transferred to 1:1 (v/v) ethanol–toluene, cleared in toluene and then embedded in paraplast. Before cutting with a MINOT microtome, the sections (15 μm) were mounted on gelatin coated glass slides. In order to unmask tissue antigens, paraplast sections were pretreated with microwave heating (5 min at 750 W) using urea 5%, Tris–HCl 0.1 M pH 9 as the antigen retrieval solution. Paraplast was removed with toluene (2 washes × 10 min each) and sections were passed through graded ethanol and finally left in distilled water. The immunostaining was performed with a modified procedure using biotin–avidin conjugate. Slices were incubated with the primary antibody (Calbindin mouse antibody, biotinilated goat anti-mouse antibody and streptavidin, from Chemicon). Endogenous peroxidase activity was suppressed by immersing the sections in a solution of 0.5% hydrogen peroxide in absolute methanol for 10 min. To prevent non-specific antibody binding, sections were incubated in a 3% normal sheep serum, 0.3% Triton X-100, Tris Buffer Saline (TBS) pH 7.4–7.5 solution for 3–4 h at RT. Then, sections were incubated in Calbindin antibody (dil: 1:400) for 48 h at room temperature (20–24 °C) followed by incubation in secondary antibody (GAM, Sigma, 1:300) for 5 h and the PAP–avidin complex, (1:400) for 3 h. Developer was performed in a 0.04% (w/v) DAB solution in buffer acetate pH 6 for 5 min, and finally sections were incubated with DAB 0.04%, hydrogen peroxide 0.01%, in acetate buffer pH 6.0, for another 5 min. The reaction was stopped by immersing the slides first in tap water and then in distilled water. Finally, sections were mounted in mounting media (DPX-Permount). Sections from each group were stained together eliminating conflicts between different experimental conditions

2.5.2. Microphotography

Calbindin immunostained sections were observed and photographed with a Zeiss Axiophot light microscope. Histological fields were randomly chosen.

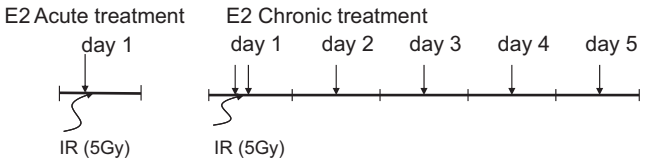

Fig. 1. Scheme of E2 treatment. Rats were treated with a subcutaneous injection of 17-β-estradiol one hour prior to 5 Gy gamma-irradiation (IR) alone (E2 acute treatment) or followed with several doses of 17-β-estradiol (1 mg/g) (E2 chronic treatment). First dose 30 min prior IR and four dairy same dose.

2.6. Motor test

2.6.1. Gait evaluation

Gait of rats from each experimental group described above were evaluated at post-irradiation day (PID) 30, 60 and 90, using an *ad hoc* quantitative test (Guelman et al., 2001). Briefly, the rats were allowed to walk freely on a non-moving paper covered horizontal surface, having their forelimbs and hindlimbs painted with two different colour inks. Both the distance between the center of each hindlimb footprint (HH: hindlimb, hindlimb) and the distance between the center of the hindlimb footprint and the ipsilateral forelimb footprint (HF: hindlimb, forelimb) taken from successive steps were measured.

2.6.2. Motor coordination

To corroborate gait data, rotarod test was performed at 30, 60 and 90 PID, with six trials for each rat on two consecutive days (three trials at intervals of 20 min/day). The apparatus (UGO Basile, Italy) was composed of an 8 cm diameter and 10 cm long bar which rotated at 13 rpm. The interval between the moment the rat was mounted on the rod until it fell off was monitored with a cutoff time of 60 s, according to the established methods, and the average time spent on the rod was calculated in C, IR, E2 and IR + E2 groups (Sakamoto et al., 2003).

2.7. Biochemical evaluation

For biochemical studies, animals were sacrificed by decapitation at different PID: 7, 10, 15 or 30 and the brains were removed. The cerebella were dissected out on an ice-cold glass petri dish. Samples were immediately weighted out, frozen and stored in liquid nitrogen until analysis.

2.7.1. PKC activity

The soluble (cytosolic fractions) and particulate fractions (membrane fractions) were obtained as previously described (Genaro and Bosca, 1993). PKC enzyme was purified by filtration through a DE 52 column (3.5 cm × 0.5 cm). The enzyme was eluted in a buffer containing 120 mM NaCl, 10 mM β-mercaptoethanol, 0.5 mM EGTA, 10 mM HEPES (pH 7.4). PKC activity was assayed on both cytosolic and membrane preparations by measuring the incorporation of ^{32}P from [$\gamma^{32}P$/ATP] into histone$_1$ (H_1). Incubations were performed for 30 min at 30 °C in a final volume of 85 μl. Final concentrations of the assay mixture were 25 μM ATP (0.4 μCi), 10 mM Mg_2 acetate, 5 mM β-mercaptoethanol, 50 mg H_1, 20 μM HEPES (pH 7.4), 0.2 μM $CaCl_2$ and 10 mg/ml phosphatidylserine vesicles, unless otherwise indicated. The incorporation of ^{32}P phosphate into H_1 was linear for at least 30 min. The reaction was stopped by the addition of 2 ml of ice-cold 5% trichloroacetic acid, 10 mM H_3PO_4. The radioactivity retained on GF/C glass-fiber filters after filtration was determined by counting the filters in 2 ml of scintillation liquid. PKC activity was determined by subtracting the incorporation of ^{32}P in the absence of calcium and phospholipids.

2.7.2. NOS activity

NOS activity was determined by measuring conversion of [U–^{14}C] arginine into [U–^{14}C] citrulline as described by Bredt and Snyder (1990). Briefly, samples were pre-incubated in 50 mM Hepes buffer pH 7.4, pre-warmed and equilibrated with 5% CO_2 in O_2. Then tissues were homogenized by sonication in 1 ml of medium containing 20 mM HEPES pH 7.4, 1 mM DTT, 1 μM leupeptin, 0.45 mM $CaCl_2$ and 0.2 mM PMSF, and incubated at 37 °C for 30 min in 5% CO_2 in O_2, in the presence of [U–^{14}C] arginine (0.5 μCi). In addition, the same buffer without $CaCl_2$ and containing 1 mM EGTA was added to another aliquot of homogenate for iNOS determination. The reaction was stopped by quick ice-cold cooling and the samples were centrifuged at 20,000 × *g* for 10 min at 4 °C. Supernatants were passed through 2 ml Dowex AG 50 WX-8 (sodium form) columns. [^{14}C] citrulline was eluted in 2 ml of water and quantified by liquid scintillation counting. Calcium dependent activity was estimated by subtracting the activity in the absence of calcium from the activity in the presence of this ion.

2.8. In vitro studies

2.8.1. Cell culture

The procedure of Gallo et al. (1982) was followed, with minor modifications. Briefly, 6–8-days-old Wistar rats were decapitated and the cerebella dissected in Krebs–Ringer solution supplemented with 2.5 g/l glucose. The meninges were eliminated, and the tissues were cut into 1 mm pieces and incubated in saline containing 0.25% trypsin, 1.2 mM SO_4Mg and 3 mg/ml bovine serum albumin for 15 min at 37 °C with continuous agitation. Digestion was stopped with 10% heat inactivated fetal bovine serum (FBS). The cells were mechanically dissociated with Pasteur pipettes of two different diameters (25 strokes) in saline solution containing 10% FBS and 0.001% DNAse. The cell suspension obtained was centrifuged at 150 × *g* for 10 min and the pellet resuspended in Basal Modified Eagle's medium supplemented with 25 mM KCl, 100 μg/ml gentamicin, 2 mM L-glutamine and 10% FBS. Cell viability was estimated using Trypan Blue exclusion and cell counting was performed in a Neubaüer camera. 106 cells (105 cells/cm^2) were seeded in 35 mm Petri dishes pre-coated with 10 μg/ml poly-D-lysine (MW > 300,000). Cell cultures were grown in a 37 °C incubator with a humidified atmosphere containing 5% CO_2. Cytosine arabinoside (ARA-C) was added at a final concentration of 10 μM 24 h after seeding to prevent glial cell growth.

2.8.2. Irradiation procedure

The γ rays source was a Theratron unit for cobalt therapy and the dose-rate was of approximately 0.4 Gy/min. The source-target distance was fixed at 95 cm. A single dose of γ-rays (0.3 Gy, the dose that produces 50% of cell death (ED50), see Guelman et al., 1996) was delivered to the dishes, randomly designed as γ-irradiated, E2/γ-irradiated and control (sham-irradiated) groups, 1–2 h after plating. Cell viability was assessed 2 days after irradiation. Each condition was run in triplicate and each experiment was repeated at least four times.

2.8.3. Viability assays

Viable neurons were identified as phase-bright cells of 6–8 μm in diameter, each with one or more neuritic processes. Cultured at this developmental age, more than 98% of the observed neurons are granule cells, according to Gallo et al. (1982). After 48 h, viable neurons were counted using Trypan Blue exclusion in five fields of triplicate samples per experimental group (100 mm^2 per dish) using a gridded ocular, under a Nikon inverted phase-contrast microscope at 200× magnification (Guelman et al., 1996). For each treatment, 200–300 cells were counted. To verify that neurons have been counted accurately, cells from several platings were tested for MTT formazan product (Zhang et al., 2003). Cells were incubated 2 h with 0.5 mg/ml MTT at 37 °C and thereafter a solution of acidic isopropanol was added. After dissolution of the blue formazan, samples were read spectrophotometrically at 570 nm.

2.8.4. ROS determination

Reactive oxygen species (ROS) levels were assessed using the method of Ceccon et al. (2000). Briefly, at 48 h post irradiation, cells were washed three times with Locke's buffer and then incubated with 10 μl of 2′,7′-dichlorofluorescein diacetate (DCFH) (1.7 mg/ml) for 15 min at 37 °C. Therefore, the cultures were washed with Locke's solution to remove extracellular DCFH-DA and 1.2 ml of 0.1 M KH_2PO_4–0.5% Triton X-100 (pH = 7) were added to the cells for 10 min at RT. Cells were scrapped from the dish and the extract was centrifuged for 5 min at 12,000 rpm (Eppendorf microfuge). Fluorescence was read at 488 nm and 525 nm (wavelengths of excitation and emission, respectively).

2.8.5. E2 treatment

A toxicity curve was performed to find the maximal non-toxic concentration of E2, being 1 μM the final concentration chosen. Half of the total E2 required was added 30 min before irradiation and the remainder 30 min after. Neuroprotective index (NI) was defined as the ratio between the percentage of the mean number of neurons or ROS levels present in E2/γ-irradiated cultures in relation to the percentage of the mean number of neurons or ROS levels present in the γ-irradiated cultures.

2.9. Statistical analysis

Data are expressed as means ± SEM from four to six independent experiments, each including three animals per treatment group (*n*). Data were analyzed using a single-factor analysis of variance (ANOVA). To test the significance of the difference between individual means a post hoc analysis of Bonferroni was performed (Hochberg and Tamhane, 1987).

3. Results

3.1. Motor evaluation

Fig. 2 shows the motor gait results. As expected according to our previous results (Zorrilla Zubilete et al., 2005) gait of PID 30 rats, after neonatal gamma-irradiation exposure, was significantly impaired, when compared to matched control animals, both in HF ($p < 0.01$) and in HH analysis ($p < 0.05$). At PID 60 HF distance was significantly impaired when compared to matched controls ($p < 0.05$). Chronic but not acute E2 treatment reverted the motor coordination deficit significantly reaching HF and HH control values at both 30 and 60 PID. Similar results to 60 PID were obtained at 90 PID (data not shown). In order to confirm the observed gait impairment another motor test was performed. A rotarod was used to evaluate motor coordination in C, IR, E2 and IR + E2 groups at 30, 60 and 90 PID. Table 1 shows that the interval of time spent on the rotarod significantly decreased in irradiated rats at all PID tested. Chronic E2 treatment improved motor coordination. Acute E2 treatment had no effect on motor

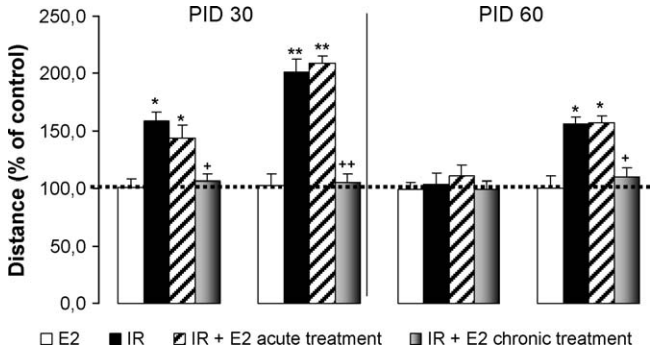

Fig. 2. Effect of perinatal in vivo E2 treatment on motor deficit induced by neonatal gamma-irradiation. Gait motor was evaluated at 30 and 60 PID in animals with a dose of 17-β-estradiol (5 mg/g) without irradiation E2 (white bars), irradiated animals without treatment IR (black bars), irradiated animals with a single dose of 17-β-estradiol (lined bars) or with a multiples doses of 17-β-estradiol (total dose = 5 mg/g) (gray bars). Results are expressed as percentage of control values. HH: distance between hindlimb footprints (control = 5.8 ± 0.5 cm); HF: distance between ipsilateral hind and forelimb footprint (control = 0.39 ± 0.06 cm) [left: HH; right: HF]. Values shown are the mean ± SEM of 7 experiments, each performed with three rats per group. $^{*}p < 0.05$, $^{**}p < 0.01$ with respect to control. $^{+}p < 0.05$, $^{++}p < 0.01$ with respect to irradiated non-treated rats.

impairment (data not shown). It is important to note that chronic 17-β-estradiol treatment of SHAM rats (E2) had not significant effect on motor gait or motor coordination (Fig. 2 and Table 1).

3.2. Cerebellar cytoarchitecture

Changes in cerebellar cytoarchitecture of irradiated rats have been demonstrated by our group (Guelman et al., 2000; Zorrilla Zubilete et al., 2005). The developmental time-course of changes in the cytoarchitecture of the cerebellar cortex from both gamma-irradiated rats and age-matched controls was studied at 7, 15, and 30 PID (see Fig. 3a–c (C), d–f (IR), g–i (IR + E2). The presence of numerous postmitotic calbindin positive neuroblasts in the external granular (EG) layer of the cerebellar cortex in non-irradiated 7-day-old rats was observed (Fig. 3a). On the contrary, the EG almost disappeared 7 days after radiation exposure (Fig. 3d) as a consequence of the effect of the ionizing noxa on developing premitotic neuroblasts. The E2 treatment (Fig. 3g) modified the abnormal morphology, and cortical cerebellar cytoarchitecture trends to have a normal stratification. At 15 and 30 PID, the thickness of cerebellar granule cell layer was notably reduced (as described in Zorrilla Zubilete et al., 2005). Purkinje cells – normally arranged in a monolayer (Fig. 3b and c) – were scattered within the cortex (see Fig. 3e and f). Fig. 3f shows that cerebellar cytoarchitecture was disrupted in 30 PID rats, the thickness of cerebellar granule cell layer was reduced, and Purkinje cells – normally arranged in a monolayer – were spread within the cortex.

Table 1
Effect of 17-β-estradiol on motor coordination abnormalities induced by neonatal gamma-irradiation. The interval of time spent on the rotarod at 13 rpm until the rats fell off was recorder for 60 s at 30, 60 and 90 PID. Six trials were performed for each group. Values represent the means ± SEM of 6 independent experiments using three rats per group.

Treatment	Time spent (s)		
	PID 30	PID 60	PID 90
Control	52.8 ± 1.2	56.7 ± 2.7	57.3 ± 3.1
E2	50.4 ± 4.3	53.5 ± 3.8	54.1 ± 4.9
IR	5.1 ± 0.8**	6.2 ± 0.4**	5.6 ± 1.9**
IR + E2	15.7 ± 0.5+	19.5 ± 0.5+	13.6 ± 3.4+

** $p < 0.01$ with respect to control.
+ $p < 0.05$ respect to irradiated without treatment.

Chronic E2 treatment was partially effective in preventing the abnormal stratification of cortical cerebellar neurons and Purkinje cells disarrangement (Fig. 3h and i). Animals treated with E2 showed similar cytoarchitecture than control (data not shown).

3.3. Cerebellar NOS activity

As previously described, after a single dose of 5 Gy of gamma-irradiation at birth, calcium dependent NOS activity was diminished compared to matched controls, reaching the maximal decrease at 15 PID and being 63% lower than control ($p < 0.001$) (Fig. 4). A progressive restoration of NOS activity was observed, returning to control values after 30 PID. Chronic treatment with E2 prevented this decrease by partially restoring NOS activity. Chronic treatment of SHAM group had no effect on NOS activity (Fig. 4). No effect was found for acute E2 treatment (data not shown).

3.4. Cerebellar PKC activity

As shown in Fig. 5, γ-rays induced an initial increment in cerebellar PKC activity that reached the maximal value at 7 PID ($p < 0.001$), returning to control values at 30 PID. Chronic but not acute treatment with 17-β-estradiol prevented PKC activity increase induced by γ-irradiation. Estradiol treatment on SHAM rats had not effect.

3.5. Neuroprotective role of E2 on irradiated cells

Table 2 shows that *in vitro* irradiation of cerebellar granule cells induced a significant decrease in cell viability ($F_{2,14} = 22{,}616$, $p < 0.001$) together with a significant increase in ROS levels (ROS: $F_{2,8} = 8882$, $p < 0.05$). E2 treatment before and after radiation exposure was effective in promoting cell viability ($p < 0.01$ when compared with control and $p < 0.05$ when compared with irradiated cultures) and in preventing the increase in ROS levels (NS when compared with control and $p < 0.05$ when compared with irradiated cultures). When the neuroprotective index was calculated (irradiated + E2/irradiated), a significant difference was observed, both in terms of viability ($F_{1,9} = 14{,}914$, $p < 0.01$) and in ROS levels ($F_{1,5} = 12{,}254$, $p < 0.05$).

4. Discussion

Ionizing radiation in biological systems induces a series of early events that occur instantly to several hours post-irradiation. Many effects involve cytoplasmatic amplification mechanisms, which include diverse signal transduction pathways (Schmidt-Ullrich et al., 2000). However, these studies have described the effect of ionizing irradiation only at very short term (between 5 and 240 min post-irradiation). We have previously performed a study (Zorrilla Zubilete et al., 2005) pointing to long-lasting effects of ionizing radiation on developing structures. A single dose of gamma-irradiation applied to the cephalic end of newborn rats induces disorganization in cerebellar cytoarchitecture correlated with motor abnormalities. The motor syndrome includes dystonia-like movements, a fine tremor and an ataxic gait. Abnormal movements are evident from 10 PID, being fully developed by 30 PID and remaining until adulthood (Zorrilla Zubilete et al., 2005). Here we show that perinatal chronic E2 treatment partially protects against radiation-induced long-term cerebellar abnormalities. In fact, E2 was able to prevent the disorganization of cerebellar cytoarchitecture and the development of the motor syndrome.

Purkinje cells are involved in the cerebellar cortex formation during neonatal life. Interestingly, estradiol contributes to Purkinje

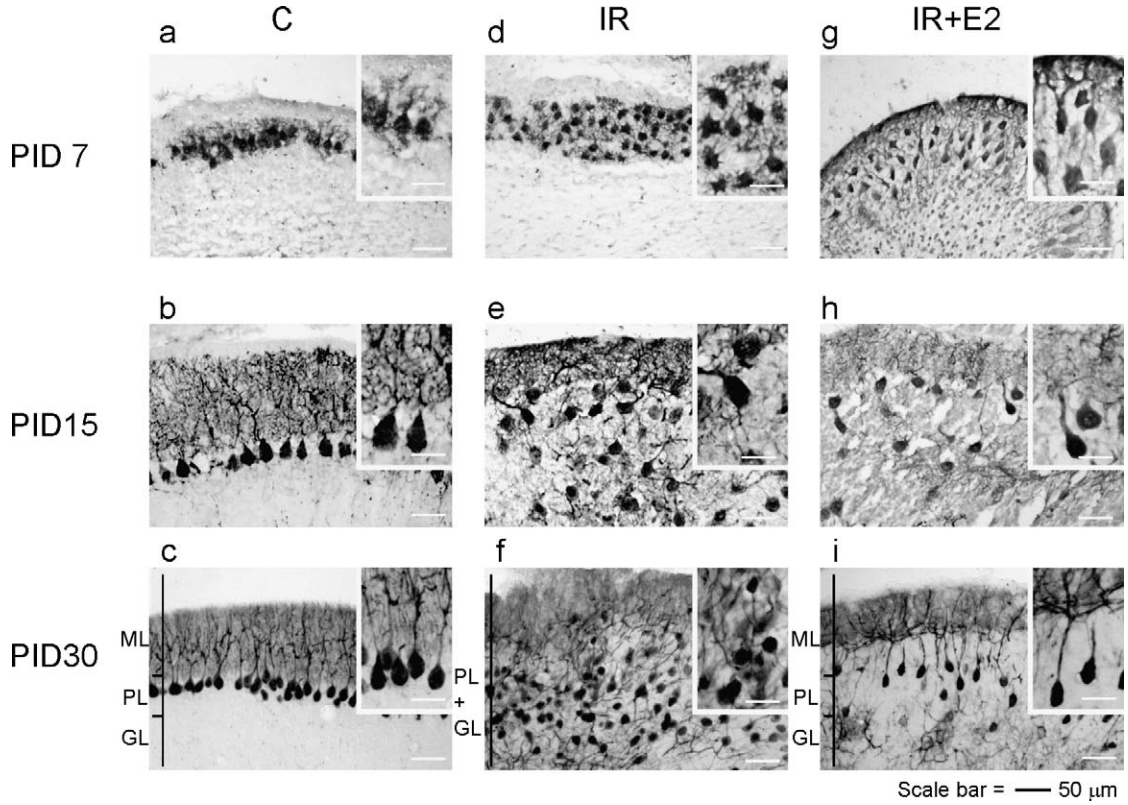

Fig. 3. Effect of perinatal in vivo 17-β-estradiol treatment on the cerebellar cortex cytoarchitecture following exposure to neonatal gamma-irradiation. Picture shown C28K Calbindin immunostaining of cerebellar cortex of parasagital sections (25 mm thick) through the cerebellar hemisphere, from animals: control (C), irradiated (IR) and irradiated + 17-β-estradiol (IR + E2) at 7 PID (a, d and g), 15 PID (b, e and h) and 30 PID (c, f and i) PID. In control three layers can be clearly distinguished: molecular layer (ML), Purkinje layer (PL) and granular layer (GL). In photo (b and d) all Purkinje cells have their primary dendrites oriented towards the pial surface and show a completely well defined dendritic tree arrangement. In irradiated cerebella a complete cytoarchitectural disorganization and shrinkage of the cerebellar cortex and agranular cortex is seen (e and f). Purkinje neurons are dispersed and their dendrites do not develop normally (3 PL + GL). In E2 treated-irradiated cerebella the general cytoarchitecture of the cortex is well preserved during temporal course of development. Most of Purkinje cells are partially arranged in a monolayer with their primary dendrite oriented towards molecular layer but some are scattered within granular layer. The correct orientation is clearly seen at 30 PID (i).

cells growth during this process (Sakamoto et al., 2003). The effect of estradiol might be mediated by estrogen receptor-β (ERβ), which is expressed in rat Purkinje cells (Price and Handa, 2000). Sakamoto (Sakamoto et al., 2003) showed in both *in vitro* and *in vivo* studies performed with newborn rats that estradiol, in the developing Purkinje cell, promotes dendritic growth and spinogenesis via ERβ. In general, free radicals generation is considered the most important mechanism involved in ionizing radiation induced neurotoxic damage. The best-known mechanism for the neuroprotective actions of E2 is related to the antioxidant properties of this hormone and its capacity to attenuate the oxidative stress-induced damage, which is independent of the activation of estrogen receptor (Behl et al., 2000). However, the mechanism(s) underlying E2 neuroprotection involve several pathways. Up-regulation of antiapoptotic proteins such as Bcl-2 (Garcia-Segura et al., 1998) and Bcl-xL (Pike, 1999) may mediate the long-term protective effects of estrogen. Recent studies suggest that physiological levels of estrogen can also rapidly modulate diverse intracellular signalling pathways (Kelly and Levin, 2001), several of which have been shown to regulate cell viability (Xia et al., 2009; Dudek et al., 1997) and may contribute to estrogen neuroprotection (Singer et al., 1999; Honda et al., 2000; Fitzpatrick et al., 2002).

Table 2
Effect of 17-β-estradiol on viability and ROS levels of irradiated cerebellar granule cells grown in vitro. Data are mean ± SEM of the percent of cell viability or ROS levels of cerebellar granule cell cultures irradiated two hours after plating when compared with control cultures (100%). Neuroprotective index refers to the ratio between irradiated + 17-β-estradiol and irradiated cultures.

	Irradiated	Irradiated + E2	Neuroprotective index
Cell viability	$43{,}832 \pm 5488^{***}$	$71{,}440 \pm 8632^{**,\S}$	$1.65 \pm 0.17^{\S\S}$
ROS levels	$144{,}067 \pm 3227^{*}$	$96{,}133 \pm 15{,}136^{\S}$	$0.66 \pm 0.09^{\S}$

* $p < 0.05$, when compared with control cultures.
** $p < 0.01$, when compared with control cultures.
*** $p < 0.001$, when compared with control cultures.
§ $p < 0.05$, when compared with irradiated cultures.
§§ $p < 0.01$, when compared with irradiated cultures.

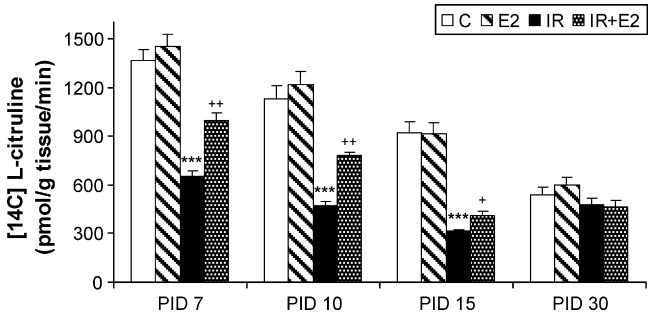

Fig. 4. Effect of perinatal in vivo 17-β-estradiol on NOS activity. NOS activity was evaluated at 7 PID, 10 PID, 15 PID and 30 PID in cerebellum from animals C: control (white bars); E2: only 17-β-estradiol chronic treatment without irradiation (bars); IR: irradiated (black bars); and irradiated plus chronic treatment of 17-β-estradiol (IR + E2) (gray bars). Values shown are mean ± SEM of 4–6 experiments, each performed with three rats per group. $^{***}p < 0.001$ with respect to control. $^{+}p < 0.05$, $^{++}p < 0.01$ with respect to irradiated non-treated rats.

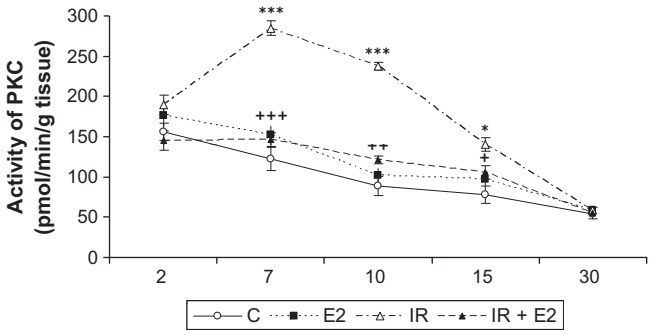

Fig. 5. Effect of perinatal in vivo 17-β-estradiol on PKC cerebellar activity. PKC activity was analyzed at 2, 7, 10, 15 and 30 post-irradiation-days (PID) in control animals (empty circle), E2 treated not-irradiated (filled square), irradiated (empty triangle) and irradiated plus multiples doses of 17-β-estradiol (5 mg/g) (filled triangle). Values shown are the means ± SEM of 4–6 experiments, each one performed with three animals per group. $^{***}p < 0.001$, $^{*}p < 0.05$ respect to control. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ with respect to irradiated without treatment animals.

PKC and NOS have been described as important enzymes in mediating cellular transduction mechanisms and in the regulation of neuronal plasticity. Previously we have shown that neonatal ionizing radiation induces an increase in PKC activity and the related decrease in nitric oxide (NO) production, indicating that NO and PKC could be intracellular events that participate in the onset of motor and cerebellar abnormalities induced by γ irradiation (Zorrilla Zubilete et al., 2005). The production of NO might lead to either toxicity or neuroprotection, depending on the level of NO, the location of NO production, the extent of oxidative stress and the type of neurodegenerative process (Chiueh, 1999; Iadecolo, 1997; Lipton, 1999). NO at low concentrations protects against peroxide-mediated toxicity (Wink et al., 1995). In fact, it has been demonstrated in vitro as well as in vivo that during a brain insult NO might be part of the physiological response to injury (Tregnago et al., 1998). In general, it is accepted that a normal pathophysiologic response of the damaged tissue involves controlled NO production and the inhibition of this response may interfere with the normal repair process. Nevertheless, a high NO production after induction of iNOS expression results in cell damage and alters the neuronal physiological function. It is important to note that, according to our results, the irradiation mainly affects microneurons such as granule and basket cells, which contain high activity of NOS (Wang et al., 1998; Schilling et al., 1994). The decrease of nNOS activity in the cerebellum is consistent with the large depletion of intrinsic nNOS expressing neurons, mainly granule cells (Wang et al., 1998; Schilling et al., 1994) after ionizing radiation. Although there is not an exact parallelism between activity and enzyme expression, as it has been shown by Yu et al. (2000) and Pelligrino et al. (1998) this specific neuronal destruction could be contributing to the diminished NOS activity. Herein we show that 17-β-estradiol chronic treatment after irradiation prevents NOS activity decrease. It has been described that at physiological levels, estrogens induce NO formation, resulting in neuroprotection (Pelligrino et al., 1998; Wen et al., 2004). In fact, Wen et al. report that both 17-β-estradiol and low concentrations of NO attenuate hydrogen peroxide (H_2O_2) induced toxicity in SK–N–SH cells, which express the (nNOS). 17-β-Estradiol rapidly induces dose-dependent activation of nNOS which may contribute to estrogens neuroprotection. A nNOS inhibitor was able to block the 17-β-estradiol induced neuroprotection. In addition, it is also noteworthy that the increase in NO induced by estrogen was small (within 2-fold) suggestive of a regulatory role rather than a toxic NO production. In accordance with this, we have demonstrated that NO produced by nNOS shows protective activity against insults that trigger tissue toxicity leading to memory impairments (Palumbo et al., 2007). It is important to note that although cerebellar cytoarchitecture and motor gait at 30 and 60 PID are abnormal, changes in NOS and PKC activities were observed as soon as 7 and 15 PID, returning to normal values by 30 PID, without E2 treatment (Zorrilla Zubilete et al., 2005). As E2 treatment reverted the early PKC and NOS alterations, it is possible to postulate that 17-β-estradiol, in this model, has a window of opportunity to exert its neuroprotective effect via NOS and PKC activities.

In order to study the role of PKC and its interdependency with NO changes, the effect of E2 on the activity of this enzyme was studied. The results shows that γ-irradiation induced an increase in PKC activity between 7 and 15 PID in irradiated rat's cerebellum, which returns to control values after 30 PID. Studies *in vitro* or in transfected cells have clearly documented that phosphorylation of constitutive NOS by different kinases, including PKC, inhibits the enzyme's catalytic activity (Bredt et al., 1992; Dinerman et al., 1994). Other authors confirmed the same effect in intact neurons (Kim et al., 2003; Riccio et al., 1996) and PC12 cells (Onoue et al., 2002). We have previously reported that γ-irradiation induces an increase in PKC activity that, in turn, contributes to a decrease in calcium dependent NOS activity. Interestingly, E2 inhibited this PKC increase. However, PKC may be involved in 17-β-estradiol action not only by its regulation of NOS activity but also by a direct effect on cells. It has been reported that PKC plays a critical role in the protective effects exerted by estrogen against β-amyloid toxicity in intact neurons, suggesting a downstream role of Bcl proteins and/or MAPK/ERK signal transduction cascades in mediating estrogen neuroprotection (Cordey et al., 2003). Although some reports suggest that PKC promotes neuronal survival, as noted above, there is also evidence that PKC can induce apoptosis or participate in the mechanisms of predominantly necrotic insults, such as glutamate excitotoxicity or ischemia (McNamara et al., 1999; Koponen et al., 2003). Differential cellular and subcellular localization of PKC isoforms are thought to account for functional specificity of enzymatic actions and contribute to different PKC effects (Mochly-Rosen, 1995). However, the opposing role of some isoforms in regulating cell survival suggests that isoforms relevant for neuroprotection may depend on the type of cell or insult studied (Chen et al., 1999; Knauf et al., 1999).

On the other hand, ROS accumulation and the consequent oxidative stress has been found as an important pathways that induce damage of cellular molecules and disturbance of normal cell function after ionizing radiation exposure (Dubner et al., 1995). Previously, we demonstrate that at very early stages, neonatal ionizing radiation exposure induces a significant increase in cerebellar ROS levels 1 h after irradiation reaching control levels at 3–5 days post-irradiation and lasting until, at least, day 90 post-irradiation (Di Toro et al., 2007). To evaluate if ROS generation could be involved in E2 neuroprotective effect we analyze the E2 effect on viability and ROS levels in culture of cerebellar granule cell. The results indicate that E2 treatment before and after radiation exposure was partially effective in preventing the fall in cell viability as well as the increase in ROS levels induced by ionizing radiation. This findings are consistent with our *in vivo* observations indicating that developing cerebellar granule cells are direct targets of ionizing radiation in the neonatal model of cephalic radiation exposure and that these changes would be partially prevented by a low concentration of E2.

In summary, 17-β-estradiol, through modulation of early increase in ROS levels and the later PKC and calcium dependent NOS activity, can protect against radiation triggered tissue toxicity and motor impairments. Finally, our results provide new evidence on the mechanisms of estrogen neuroprotection. Extensive understanding of estrogen actions is particularly important for

the development of therapeutic strategies against the adverse consequences of radiotherapy.

Acknowledgements

We wish to thank the personnel from the Center of Radiotherapy De la Torre, Buenos Aires, Argentina, for their assistance with the irradiation of the animals and María Rosa Gonzalez Murano from the National Council of Scientific and Technical Research (CONICET), Buenos Aires, Argentina, for her careful and skillful technical assistance. This work was supported by grants from the University of Buenos Aires (M040) to LMZ and CONICET (PIP 00281) to AMG.

References

Almli, L.M., Hamrick, S.E.G., Koshy, A.A., Täuber, M.G., Ferriero, D.M., 2001. Multiple pathways of neuroprotection against oxidative stress and excitotoxic injury in immature primary hippocampal neurons. Dev. Brain Res. 132, 121–129.

Behl, C., Moosmann, B., Manthey, D., Heck, S., 2000. The female sex hormone oestrogen as neuroprotectant: activities at various levels. Novartis Found Symp. 230, 221–234 discussion 234–238.

Bredt, D.S., Ferris, C.D., Snyder, S.H., 1992. Nitric oxide synthase regulatory sites: phosphorylation by cyclic AMP-dependent protein kinase, protein kinase C, and calcium/calmodulin protein kinase: identification of flavin and calmodulin binding sites. J. Biol. Chem. 267, 10976–10981.

Bredt, D.S., Snyder, S.H., 1990. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc. Natl. Acad. Sci. U.S.A. 87, 682–685.

Ceccon, M., Giusti, P., Facci, L., Borin, G., Imbesi, M., Floreani, M., Skaper, S.D., 2000. Intracellular glutathione levels determine cerebellar granule neuron sensitivity to excitotoxic injury by kainic acid. Brain Res. 862, 83–89.

Chen, J., Zhang, S., Zuo, P., Tang, L., 1997. Memory-related changes of nitric oxide synthase activity and nitrite level in rat brain. Neuroreports 8, 1771–1774.

Chen, N., Ma, W., Huang, C., Dong, Z., 1999. Translocation of protein kinase Cepsilon and protein kinase Cdelta to membrane is required for ultraviolet B-induced activation of mitogen-activated protein kinases and apoptosis. J. Biol. Chem. 274 (15), 389–415 394.

Chiueh, C.C., 1999. Neuroprotective properties of nitric oxide. Ann. N. Y. Acad. Sci. 890, 301–311.

Cordey, M., Gundimeda, U., Gopalakrishna, R., Pike, C.J., 2003. Estrogen activates protein kinase C in neurons: role in neuroprotection. J. Neurochem. 84, 1340–1348.

Cordey, M., Pike, C.J., 2006. Conventional protein kinase C isoforms mediate neuroprotection induced by phorbol ester and estrogen. J. Neurochem. 96 (1), 204–217.

Datta, R., Kojima, H., Yoshida, K., Kufe, D., 1997. Caspase-3-mediated cleavage of protein kinase C theta in induction of apoptosis. J. Biol. Chem. 272, 20317–20320.

Dawson, T.M., Snyder, S.H., 1994. Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J. Neurosci. 14, 5147–5159.

Dawson, V.L., Dawson, T.M., 1996. Nitric oxide neurotoxicity. J. Chem. Neuroanat. 10, 179–190.

Dinerman, J.L., Steiner, J.P., Dawson, T.M., Dawson, V.L., Snyder, S.H., 1994. Cyclic nucleotide dependent phosphorylation of neuronal nitric oxide synthase inhibits catalytic activity. Neuropharmacology 33, 1245–1251.

Di Toro, C.G., Di Toro, P.A., Zieher, L.M., Guelman, L.R., 2007. Sensitivity of cerebellar glutathione system to neonatal ionizing radiation exposure. Neurotoxicology 28 (3), 555–561.

Dopico, A.M., Zieher, L.M., Mayo, J., Altschuller, N., Lopez, J.J., Jaim-Etcheverry, G., 1989. Long term changes in noradrenaline (NA) and dopamine (DA) contents of rats cerebellum following neonatal X-irradiation. Neurochem. Int. 15, 97–105.

Dubner, D., Gisone, P., Jaitavich, I., Perez, M., 1995. Free radicals production and estimation of oxidative stress related to gamma irradiation. Biol. Trace. Elem. Res. 47, 265–270.

Dudek, H., Datta, S.R., Franke, T.F., Birnbaum, M.J., Yao, R., Cooper, G.M., Segal, R.A., Kaplan, D.R., Greenberg, M.E., 1997. Regulation of neuronal survival by the serine-threonine proteinkinase Akt. Science 275, 661–666.

Eshhar, N., Striem, S., Kohen, R., Tirosh, O., Biegon, A., 1995. Neuroprotective and antioxidant activities of HU-211, a novel NMDA receptor antagonist. Eur. J. Pharmacol. 283, 19–29.

Fitzpatrick, J.L., Mize, A.L., Wade, C.B., Harris, J.A., Shapiro, R.A., Dorsa, D.M., 2002. Estrogen-mediated neuroprotection against beta-amyloid toxicity requires expression of estrogen receptoralpha or beta and activation of the MAPK pathway. J. Neurochem. 82, 674–682.

Gallo, V., Ciotti, M.T., Coletti, A., Aloisi, F., Levi, G., 1982. Selective release of glutamate from cerebellar granule cells differentiating in culture. Proc. Natl. Acad. Sci. 79, 7919–7923.

Garcia-Segura, L.M., Cardona-Gomez, P., Naftolin, F., Chowen, J.A., 1998. Estradiol upregulates Bcl-2 expression in adult brain neurons. Neuroreport 9, 593–597.

Garcia-Segura, L.M., Azcoitia, I., DonCarlos, L.L., 2001. Neuroprotection by estradiol. Prog. Neurobiol. 63 (1), 29–60.

Genaro, A.M., Bosca, L., 1993. Early signals in alloantigen induced B-cell proliferation. Comparison between B-cell triggering by intact allogeneic cell and solubilized alloantigen. J. Immunol. 151 (4), 1832–1843.

Gonzalez-Aguilar, F., De Robertis, E., 1963. A formalin-perfusion metod for histophysiological study of the central nervous system with the electron microscopy. Neurology 13, 758–763.

Green, P.S., Simpkins, J.W., 2000. Neuroprotective effects of estrogens: potential mechanisms of action. Int. J. Dev. Neurosci. 18, 347–358.

Guelman, L.R., Cabana, J.I., Pagotto, R.M.L., Zieher, L.M., 2005. Ionizing radiation-induced damage on developing cerebellar granule cells cultures can be prevented by an early amifostine post-treatment. Int. J. Dev. Neurosci. 23 (1), 1–7.

Guelman, L.R., Zieher, L.M., Fiszman, M.L., 1996. The effect of x-radiation on cerebellar granule cells grown in culture. Ganglioside GM1 neuroprotective activity. Neurochem. Int. 29, 521–527.

Guelman, L.G., Zorrilla Zubilete, M.A., Rios, H., Dopico, A.M., Zieher, L.M., 2000. GM1 ganglioside treatment protects against long-term neurotoxic effects of neonatal X-irradiation on cerebellar cortex cytoarchitecture and motor function. Brain Res. 858 (2), 303–311.

Guelman, L.R., Zorrilla Zubilete, M.A., Ríos, H., Di Toro, C.G., Dopico, A.M., Zieher, L.M., 2001. Motor, cytoarchitectural and biochemical assessment of pharmacological neuroprotection against CNS damage induced by neonatal ionizing radiation exposure. Brain Res. Prot. 7 (3), 203–210.

Guelman, Laura Ruth, Pagotto, Romina María del Luján, Di Toro, Cecilia Gabriela, Zieher, Luis María, 2004. Deferoxamine antioxidant activity on cerebellar granule cells gamma-irradiated in vitro. Neurotoxicol. Teratol. 26, 477–483.

Hawkins, H.S., Son, H., Arancio, O., 1998. Nitric oxide as a retrograde messenger during long-term potentiation in hippocampus. Prog. Brain Res. 188, 155–172.

Henderson, V.W., Paganini-Hill, A., Emanuel, C.K., Dunn, M.E., Buckwalter, J.G., 1994. Estrogen replacement therapy in older women. Comparisons between Alzheimer's disease cases and nondemented control subjects. Arch. Neurol. 51, 896–900.

Hochberg, Y., Tamhane, A., 1987. Multiple Comparison Procedures. Wiley, New York.

Honda, K., Sawada, H., Kihara, T., Urushitani, M., Nakamizo, T., Akaike, A., Shimohama, A., 2000. Phosphatidylinositol 3-kinase mediates neuroprotection by estrogen in cultured cortical neurons. J. Neurosci. Res. 60, 321–327.

Iadecolo, C., 1997. Bright and dark sides of nitric oxide in ischaemic brain injury. Trends Neurosci. 20, 132–139.

Ishikawa, Y., Satoh, T., Enokido, Y., Nishio, C., Ikeuchi, T., Hatanaka, H., 1999. Generation of reactive oxygen species, release of L-glutamate and activation of caspases are required for oxygen-induced apoptosis of embryonic hippocampal neurons in culture. Brain Res. 824, 71–80.

Kelly, M.J., Levin, E.R., 2001. Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol. Metab. 12, 152–156.

Kim, H., Sasaki, T., Maeda, K., Koya, D., Kashiwagi, A., Yasuda, H., 2003. Protein kinase C beta selective inhibitor LY333531 attenuates diabetic hyperalgesia through ameliorating cGMP level of dorsal root ganglion neurons. Diabetes 52 (8), 2102–2109.

Knauf, J., Elisei, R., Mochly-Rosen, D., Liron, T., Chen, X.N., Gonsky, R., Korenberg, J.R., Fagin, J.A., 1999. Involvement of protein kinase Cepsilon (PKCepsilon) in thyroid cell death. A truncated chimeric PKCepsilon cloned from a thyroid cancer cell line protects thyroid cells from apoptosis. J. Biol. Chem. 274 (23), 414–423 425.

Koponen, S., Kurkinen, K., Akerman, E., Mochly-Rosen, D., Chan, P.H., Koistinaho, J., 2003. Prevention of NMDA-induced death of cortical neurons by inhibition of protein kinase C zeta. J. Neurochem. 86, 442–450.

Lee, S.L., Mc Ewen, B.S., 2001. Neurotrophic and neuroprotective actions of estrogens and their therapeutic implications. Annu. Rev. Pharmacol. Toxicol. 41, 569–591 Review.

Lev-Ram, V., Makings, L.R., Keitz, P.F., Kao, J.P., Tsien, R.Y., 1995. Longterm depression in cerebellar Purkinje neurons results from coincidence of nitric oxide and depolarization-induced Ca^{2+} transients. Neuron 15 (2), 407–415.

Lipton, S.A., 1999. Neuronal protection and destruction by NO (review). Cell Death Differ. 6, 943–951.

Liu, H., McPherson, H.C., Yao, Z., 2001. Preconditioning attenuates apoptosis and necrosis: role of protein kinase C epsilon and delta isoforms. Am. J. Physiol. Heart Circ. Physiol. 281, H404–H410.

Maher, P., 2001. How protein kinase C activation protects nerve cells from oxidative stress-induced cell death. J. Neurosci. 21, 2929–2938.

McNamara, R.K., Wees, E.A., Lenox, R.H., 1999. Differential subcellular redistribution of protein kinase C isozymes in the rat hippocampus induced by kainic acid. J. Neurochem. 72, 1735–1743.

Mellor, H., Parker, P.J., 1998. The extended protein kinase C superfamily. Biochem. J. 332, 281–292.

Mochly-Rosen, D., 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. Science 268, 247–251.

Nakane, M., Mitchell, J., Forstermann, U., Murad, F., 1991. Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. Biochem. Biophys. Res. Commun. 180, 1396–1402.

Onoue, S., Endo, K., Yajima, T., Kashimoto, K., 2002. Pituitary adenylate cyclase activating polypeptide regulates the basal production of nitric oxide in PC12 cells. Life Sci. 71 (2), 205–214.

Palumbo, M.L., Fosser, N.S., Rios, H., Zorrilla Zubilete, M.A., Guelman, L.R., Cremaschi, G.A., Genaro, A.M., 2007. Loss of hippocampal neuronal nitric oxide synthase contributes to the stress-related deficit in learning and memory. J. Neurochem. 102, 261–274.

Pelligrino, D.A., Santizo, R., Baughman, V.L., Wang, Q., 1998. Cerebral vasodilating capacity during forebrain ischemia: effects of chronic estrogen depletion and repletion and the role of neuronal nitric oxide synthase. Neuroreport 9, 3285–3291.

Pike, J.C., 1999. Estrogen modulates neuronal Bcl-xL expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. J. Neurochem. 72, 1552–1563.

Price, R.H., Handa, R.J., 2000. Expression of estrogen receptor-beta protein and mRNA in the cerebellum of the rat. Neurosci. Lett. 288, 115–118.

Riccio, A.E., Esposito, M.L., Eboli, M., 1996. Modulation by protein kinase C of nitric oxide and cyclic GMP formation in cultured cerebellar granule cells. Brain Res. 718, 159–164.

Sakamoto, H., Mezaki, Y., Shikimi, H., Ukena, K., Tsutsui, T., 2003. Dendritic growth and spine formation in response to estrogen in the developing Purkinje cell. Endocrinology 144, 4466–4477.

Schilling, K., Schmidt, H.H., Baader, S.L., 1994. Nitric oxide synthase expression reveals compartments of cerebellar granule cells and suggests a role for mossy fibers in their development. Neuroscience 59 (4), 893–903.

Schmidt-Ullrich, R.K., Dent, P., Grant, S., Mikkelsen, R.B., Valerie, K., 2000. Signal transduction and cellular radiation responses. Radiat. Res. 153, 245–257.

Singer, C.A., Figueroa-Masot, X.A., Batchelor, R.H., Dorsa, D.M., 1999. The mitogen-activated protein kinase pathway mediates estrogen neuroprotection after glutamate toxicity in primary cortical neurons. J. Neurosci. 19, 2455–2463.

Tregnago, M., Virgili, M., Monti, B., Guamieri, T., Constestabile, A., 1998. Alteration of neuronal nitric oxide synthase activity and expression in the cerebellum and forebrain of microencephalic rats. Brain Res. 793, 54–60.

Walton, M., Woodgate, A.M., Muravlev, A., Xu, R., During, M.J., Dragunow, M., 1999. CREB phosphorylation promotes nerve cell survival. J. Neurochem. 73, 1836–1842.

Wang, M., Nayakama, T., Inuoe, N., Kato, T., 1998. Quantitative analysis of nitric oxide synthase expressed in developing and differentiating rat cerebellum. Dev. Brain Res. 111, 65–75.

Wen, Y., Perez, E.J., Green, P.S., Sarkar, S.N., Simpkins, J.W., 2004. nNOS is involved in estrogen mediated neuroprotection in neuroblastoma cells. Neuroreport 15 (9), 1515–1518.

Wink, D.A., Cook, J.A., Pacelli, R., Liebmann, J., Krishna, M.C., Mitchell, J.B., 1995. Nitric oxide (NO) protects against cellular damage by reactive oxygen species. Toxicol. Lett. 82/83, 221–226.

Xia, Y., Xing, J.Z., Krukoff, T.L., 2009. Neuroprotective effects of R,R-tetrahydrochrysene against glutamate-induced cell death through anti-excitotoxic and anti-oxidant actions involving estrogen receptor-dependent and -independent pathways. Neuroscience 162 (2), 292–306.

Xie, H., Guo, Q., Zhu, H., Wooten, M.W., Mattson, M.P., 2000. Protein kinase C iota protects neural cells against apoptosis induced by amyloid beta-peptide. Mol. Brain Res. 82, 107–113.

Yu, W.J., Juang, S.W., Lee, J.J., Liu, T.P., Cheng, J.T., 2000. Decrease of neuronal nitric oxide synthase in the cerebellum of aged rats. Neurosci. Lett. 291, 37–40.

Yun, H.Y., Dawson, V.L., Dawson, T.M., 1997. Nitric oxide in health and disease of the nervous system (review). Mol. Psychiatry 2, 300–310.

Zandi, P.P., Carlson, M.C., Plassman, B.L., Welsh-Bohmer, K.A., Mayer, L.S., Steffens, D.C., Breitner, J.C., 2002. Cache Hormone replacement therapy and incidence of Alzheimer disease in older women: the Cache county study. County memory study investigators. JAMA 2882, 123–129.

Zhang, J., Geula, C., Lu, C., Koziel, H., Hatcher, L.M., Roisen, F.J., 2003. Neurotrophins regulate proliferation and survival of two microglial cell lines in vitro. Exp. Neurol. 183, 469–481.

Zorrilla Zubilete, M.A., Ríos, H., Silberman, D.M., Guelman, L.R., Ricatti, J.M., Genaro, A.M., Zieher, L.M., 2005. Altered nitric oxide synthase and PKC activities in cerebellum of gamma-irradiated neonatal rats. Brain Res. 1051 (1-2), 8–16.