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ORIGINAL ARTICLE

An early treatment with 17-β-estradiol is neuroprotective against the long-term effects of neonatal ionizing radiation exposure

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Abstract

Ionizing radiations can induce oxidative stress on target tissues, acting mainly through reactive oxygen species (ROS). The aim of this work was to investigate if 17-β-estradiol (βE) was able to prevent hippocampal-related behavioral and biochemical changes induced by neonatal ionizing radiation exposure and to elucidate a potential neuroprotective mechanism. Male Wistar rats were irradiated with 5 Gy of X-rays between 24 and 48 h after birth. A subset of rats was subcutaneously administered with successive injections of βE or 17-α-estradiol (αE), prior and after irradiation. Rats were subjected to different behavioral tasks to evaluate habituation and associative memory as well as anxiety levels. Hippocampal ROS levels and protein kinase C (PKC) activity were also assessed. Results show that although βE was unable to prevent radiation-induced hippocampal PKC activity changes, most behavioral abnormalities were reversed. Moreover, hippocampal ROS levels in βE-treated irradiated rats approached control values. In addition, αE administered to irradiated animals was effective in preventing radiation-induced alterations. In conclusion, βE was able to counteract behavioral and biochemical changes induced in irradiated animals, probably acting through an antioxidant mechanism.

Keywords: 17-β-estradiol, behavior, hippocampus, ionizing radiation, PKC, ROS.

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It is widely known that ionizing radiation is a physical agent broadly used to kill tumor cells in human cancer therapy. Unfortunately, during the treatment of cerebral tumors, normal brain can undergo undesirable tissue injury. Moreover, harmful effects on central nervous system (CNS) have been observed in children exposed to radiation at the time of Hiroshima and Nagasaki atomic explosions as well as during the Chernobyl accident (UNSCEAR 1993; Kimler 1998), suggesting a high radiosensitivity of developing brain.

As reported by different authors, ionizing radiations are capable to induce oxidative stress on target tissues, acting mainly through the generation of a type of spontaneously generated radicals, ROS (Halliwell 2006), a common mechanism in animal models of neurodegenerative diseases (Cassarino and Bennett 1999) and neurotoxicity (Goodlett and Horn 2001). In particular, previous studies from our laboratory (Guelman *et al.* 1993, 2001, 2003; Di Toro *et al.* 2007; Caceres *et al.* 2009, 2010) and from others (Riley 1994; Limoli *et al.* 2004) have shown that the developing cerebellum (CE) and hippocampus (Hip) are structures that can be damaged by radiation-induced ROS. Moreover, CE- and Hip-related behavioral impairments have been found after neonatal X irradiation (Caceres *et al.* 2009, 2010).

The elevated vulnerability of the immature brain, together with the limited availability of therapeutic tools to attenuate

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Abbreviations used: βE, 17-β-estradiol; αE, 17-α-estradiol; CβE, control + βE; C, control + vehicle; CE, cerebellum; DG, dentate gyrus; EPM, elevated plus maze; ER, estrogen receptors; Hip, hippocampus; IA, inhibitory avoidance; OF, open field; ROS, reactive oxygen species; Rx, irradiated + vehicle; RxαE, irradiated + αE.

CNS damage, supports the search and development of new preventive and therapeutic strategies (Manthey and Behl 2006). Previous data of our laboratory demonstrated that it is possible to partially prevent cerebellar-related behavioral, oxidative, and histological damage induced by ionizing radiations through the use of chemical agents, such as amifostine and deferoxamine that could be neuoprotective by acting through an antioxidant mechanism (Guelman *et al.* 2003, 2004, 2005).

Interestingly, sex hormones, such as estrogens have shown to exert a neuroprotective role in animal models of focal and global cerebral ischemia (Wise and Dubal 2000; Lebesgue *et al.* 2009; Saraceno *et al.* 2010). Moreover, the treatment of male rats with βE improved neurological outcome following traumatic injury (Green and Simpkins 2000) and neonatal stroke (Nuñez *et al.* 2007). Further, neuronal loss produced in the dentate gyrus (DG) of the Hip after systemic administration of the neurotoxin kainic acid was significantly reduced by pre-treatment with βE (Azcoitia *et al.* 1998; Behl 2002; Hilton *et al.* 2004). Finally, βE has been shown to attenuate neural injury because of seizures (Velísková *et al.* 2000) and excitotoxicity (Huang *et al.* 2004). Therefore, it would be hypothesized that βE could prevent the damage induced by different insults whose pathophysiology involves the generation of oxidative stress, including ionizing radiations. In fact, recently published data of our laboratory showed that βE administration was able to prevent CE-associated changes in neonatally irradiated rats (Zorrilla Zubilete *et al.* 2011).

Although it would not be discarded that βE could be neuroprotective acting through the activation of estrogen receptors (ER), the antioxidant activity of estrogens has received increasing attention (Behl 2002; Shughrue *et al.* 1997; Jover *et al.* 2002; Kii *et al.* 2005; Andreescu *et al.* 2007; Connell *et al.* 2007). In fact, it has been suggested that estrogens have antioxidant properties that are related to the presence of a hydroxyl group at the C3 position on the steroid A ring of the hormone (Mann *et al.* 2007). It is important to note that direct antioxidant effects of βE were observed in the absence of functional ER, *in vitro* and *in vivo* (Prokai and Simpkins 2007; McClean and Nuñez 2008), although indirect mechanisms, such as up-regulation of antioxidant enzymes (Borrás *et al.* 2005) or interaction with lipid-transport proteins to prevent membrane lipid peroxidation should not be discarded (Tikkanen *et al.* 2002). Moreover, we found that while an increase in ROS levels was observed in irradiated cerebellar granule cells cultures, a re-establishment of oxidative state was achieved after βE treatment, suggesting that an antioxidant action could underlie βE neuroprotection (Zorrilla Zubilete *et al.* 2011).

Accordingly, it is reasonable to hypothesize that if βE acts through an antioxidant mechanism, the use of non-feminizing enantiomers of estrogens, like α-estradiol (αE), can also be potently neuroprotective *in vitro* and in animal models, resulting therefore clinically relevant (Simpkins *et al.* 2004). Supporting this, Yi *et al.* (2008) demonstrated that treatment with either the naturally occurring estrogen isomer αE (mostly inactive on the ER) or the active isomer βE, markedly reduced ischemic brain damage produced by middle cerebral artery occlusion in ovariectomized rats, supporting the hypothesis that neuroprotection by βE would be reached through an antioxidant mechanism.

Nevertheless, since various reports demonstrate that ER could be involved in the neuroprotective actions of estradiol (Garcia-Segura *et al.* 2001; Toran-Allerand *et al.* 2005; Prokai and Simpkins 2007; Morissette *et al.* 2008), the role of ER-dependent mechanisms would not be ignored. Interestingly, at present, it is believed that ERβ plays a crucial role in brain development and maintenance, whereas ERα seems to be implicated in a variety of cellular events associated with neuroprotection against different neuronal insults (Dubal and Wise 2001; Zhang *et al.* 2009). However, the role of each ER in neuroprotection appears to be dependent on the cellular parameter analyzed, the type of injury, and the location of the damage.

Estrogen receptors are abundantly expressed in many brain areas (DonCarlos *et al.* 2009). Although Hip has fewer receptors that other areas, the existence of hippocampal ER should not be underestimated (Shughrue *et al.* 1997; Toran-Allerand 2004; Rhodes and Frye 2006; Fan *et al.* 2010).

In consequence, the aim of this work was to investigate if βE was able to prevent the hippocampal-related behavioral and biochemical changes induced by neonatal ionizing radiation. To elucidate a possible mechanism of the neuroprotective effect, an isomer that hardly binds *in vivo* to ER, αE, was administered to irradiated animals.

Materials and methods

Animals

Pregnant albino Wistar females were bred in our colony and isolated a few days before delivery. The day of birth (day 0) was known by daily inspection of the cages. Neonatal male rats were randomly separated into six groups: (a) control + vehicle (C), (b) control + βE (CβE), (c) control + αE (CαE), (d) irradiated + vehicle (Rx), (e) irradiated + βE (RxβE), and (f) irradiated + αE (RxαE). Pups were irradiated or sham irradiated; rats from groups (b) and (e) were treated with βE and rats from groups (c) and (f) were treated with αE. All animals were kept with their dams until 22 days of age. After weaning, they were separated into their respective groups and maintained four per cage until 30 days, with food and water *ad libitum*, on 12 h light–dark cycles (lights on at 7 AM) at 22 ± 2°C.

Animals were handled and killed according to the Institutional Committee for the Use and Care of Laboratory Animal rules (CICUAL, School of Medicine, University of Buenos Aires, Argentina) and the protocol was approved by this Committee under resolution 2079/07. The CICUAL adheres to the rules of the 'Guide for the Care and Use of Laboratory Animals' (NIH) and the institution has an Animal Welfare Assurance approved by the Public

Health Service with the assurance number A5801-01. Adequate measures were taken to minimize animal pain or discomfort. Moreover, the ARRIVE guidelines have been followed (Kilkenny *et al.* 2010).

For all experimental procedures, male rats of 30 days were used. For some behavioral tasks, the animal was re-exposed to the behavioral device 1 h after the first session. After these studies, animals were killed in a euthanasia CO_2 chamber. For biochemical studies, rats were killed by decapitation with guillotine.

For all studies, seven to eight animals of each experimental group were used (total animals used: 132).

Irradiation procedure

Only the heads of neonatal male Wistar rats (between 24 and 48 h after birth) were exposed to a single 5 Gy dose of X-rays, obtained from a high-energy electron linear accelerator (Mevatron Siemens, MO, USA, 6 MV of photon energy). The dose-rate used was of approximately 1 Gy/min, being the total time of radiation exposure around 5 min. Radiation energy absorbed into the tissue was approximately 5 J/kg, since 1 Gy is a unit of absorbed dose equivalent to 1 J/kg. Pups were immobilized in a plastic holder and the heads fixed in a plastic frame. The beam of X-rays was collimated upon the head at a distance of 50 cm, and homogenized using acrylic plates. The number of subjects remained unchanged throughout the study, since at the dose of radiation used in this study, no animal deaths were produced. Radiation protection was always used by the experimenter.

Pharmacological treatment

Administration of 17β- and 17α-estradiol. Five successive injections of 50 μg/kg each βE and αE (Sigma-Aldrich, St Louis, MO, USA) were administered subcutaneously to rats from groups (b) and (e) (βE), and (c) and (f) (αE). The first dose was administered 30 min before irradiation and the second was injected immediately after the irradiation; after that, three consecutive daily doses were administered to rat pups. Rats not receiving estradiol [groups (a) and (d), C and Rx rats] were injected with vehicle (0.9% sodium chloride in distilled water), leaving an interval of 30 min between the first and second injection, and after that, three consecutive daily doses of vehicle were administered to rat pups.

Behavioral testing

All behavioral experiments were carried out in an isolated behavioral room, between 11 AM and 4 PM.

Open field task (OF)

Habituation to a novel environment is believed to be one of the most elementary forms of non-associative learning, known to depend on Hip (Vianna *et al.* 2000), in which the repeated exposure to the same environment induces a reduction in the exploratory behavior.

Experiments were carried out as described in Caceres *et al.* (2010). The number of lines crossed was recorded using a digital video camera and compared with the first session to evaluate habituation memory (Barros *et al.* 2006).

Elevated plus maze

The elevated plus maze (EPM) is a test used as a measure of anxiety, based on the natural conflict between the drive to explore a new environment and the tendency to avoid a potentially dangerous area (Pellow *et al.* 1985). The device was a wooden custom-made maze and consisted of four arms of equal dimensions (50 cm × 10 cm), raised 50 cm above the floor. Two arms, enclosed by walls of 40 cm high, were perpendicular to two opposed open arms. To avoid falls, the open arms were surrounded by a Formica rim of 0.5 cm. The EPM was dimly illuminated with a lamp located 200 cm above the maze.

When the session started, the rats were placed in the center of the maze, facing one of the closed arms. The session was recorded for 5 min using a digital video camera.

The percent of time spent on each arm was scored. Maze was cleaned between sessions with a 10% alcohol solution (Brenes *et al.* 2009).

Some rats fell down when walking in open arms. These animals were excluded from the study.

Inhibitory avoidance

Inhibitory avoidance (IA) task measures the memory of an aversive experience through the simple avoidance of a location in which the unpleasant experience occurred. This task depends heavily on the dorsal Hip (Ennaceur and Delacour 1988; Izquierdo and Medina 1997). We used an IA apparatus as described by Roozendaal *et al.* (2002). Experiments were conducted as described in Caceres *et al.* (2010).

ROS determination

The levels of hippocampal ROS were determined by a method described by Driver *et al.* (2000) and modified by Caceres *et al.* (2010).

PKC activity

Hippocampi were quickly dissected in ice and processed for PKC activity as described in Zorrilla Zubilete *et al.* (2005).

Statistical analysis

The results were analyzed using the statistical package SigmaStat 3.5 (Systat Software, Inc., San Jose, CA, USA). Significant differences between groups were determined by two-way ANOVA statistical analysis and Tukey test was applied for *post hoc* comparisons. Results are expressed as mean values ± SEM of seven to eight animals. A probability < 0.05 was accepted as significant.

Results

Data show that the increase in the escape latency in the IA task observed in irradiated animals was not significantly reversed by the administration of βE ($F_{1,32} = 12.990$, $p < 0.001$; Rx vs. C, $p < 0.01$; RxβE vs. CβE, $p < 0.05$; Rx vs. RxβE, NS, Fig. 1). Similarly, the activity of hippocampal PKC, a protein involved in associative memory that was increased after neonatal ionizing radiation exposure, was also unchanged after βE treatment ($F_{1,19} = 19.345$, $p < 0.001$; Rx vs. C, $p < 0.01$; RxβE vs. CβE, $p < 0.01$; Rx vs. RxβE, NS, Fig. 2).

Data in Fig. 3 show that the deficit in habituation memory induced in 30-day-old rats exposed neonatally to ionizing

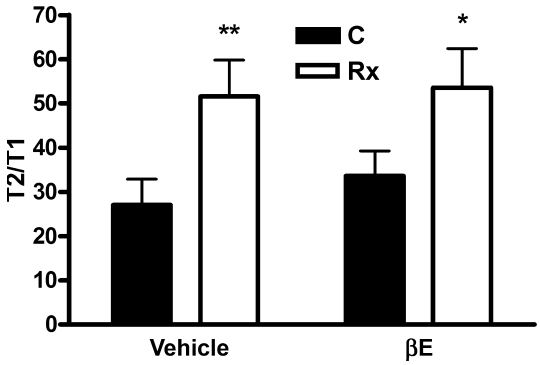

Fig. 1 Latency to enter the dark compartment in the inhibitory avoidance task. Filled bars: control rats (C); open bars: irradiated rats (Rx). βE: 17-β-estradiol. $*p < 0.05$ and $**p < 0.01$ respect to respective control. Data are mean of the ratio of the latency to enter the dark compartment between retention and training sessions ± SEM.

radiation, measured in the OF task, was prevented by βE administration without affecting control performance ($F_{1,56}$ = 6.326, $p < 0.001$; first session vs. second session: C, $p < 0.001$; Rx, NS; RxβE, $p < 0.001$).

Likewise, Fig. 4 shows that the time spent in open arms in the EPM task was increased in irradiated animals when compared to the respective control ($F_{1,26}$ = 10.429, $p < 0.01$; Rx vs. C, $p < 0.001$). βE was effective in reversing this behavior, resulting statistically similar to control values and significantly different from irradiated animals (RxβE vs. Rx, $p < 0.01$).

Finally, increased hippocampal ROS levels induced by radiation exposure ($F_{1,32}$ = 11.025, $p < 0.001$; C vs. Rx, $p < 0.001$) were fully restored in βE-treated irradiated animals (Rx vs. RxβE, $p < 0.001$), approaching to control values (Fig. 5). Based on these results, it will be postulated that the observed reversion of hippocampal oxidative stress achieved by βE could underlie estrogen neuroprotection of

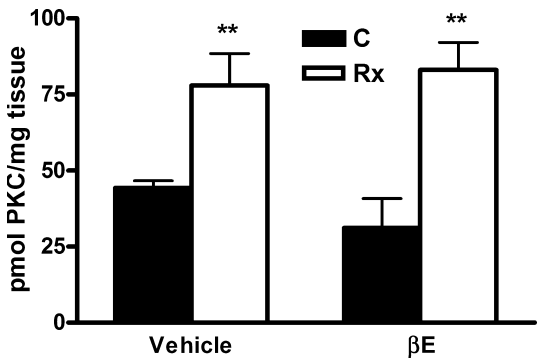

Fig. 2 Hippocampal PKC activity. Filled bars: control rats (C); open bars: irradiated rats (Rx). βE: 17-β-estradiol. $**p < 0.01$ respect to respective control. Data are mean of pmol PKC/mg tissue ± SEM.

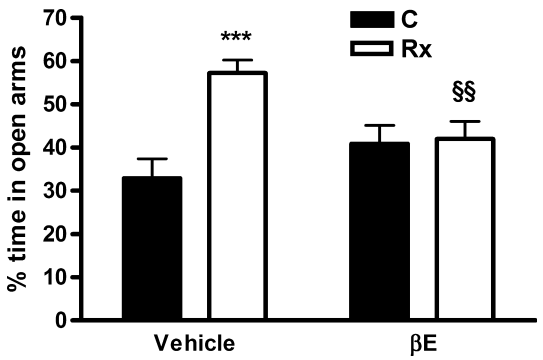

Fig. 4 Percent time spent in open arms in the elevated plus maze task. Filled bars: control rats (C); open bars: irradiated rats (Rx). βE: 17-β-estradiol. $***p < 0.001$ respect to respective control rats; §§$p < 0.01$ respect to vehicle-injected, Rx rats. Data are mean of the percent of time spent in open arms respect to total time ± SEM.

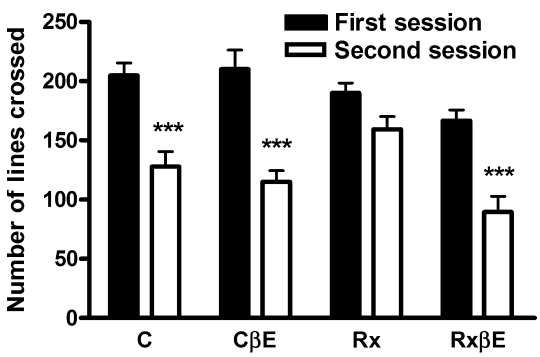

Fig. 3 Number of lines crossed in the open field task. Filled bars: first session; open bars: second session. C: control rats; CβE: β-estradiol-treated control rats; Rx: irradiated rats; RxβE: β-estradiol-treated irradiated rats. $***p < 0.001$ respect to the first session. Data are mean of the number of lines crossed ± SEM.

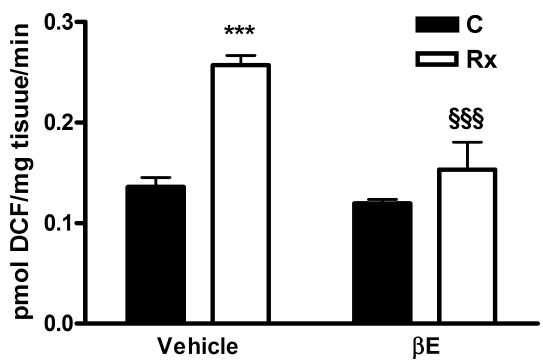

Fig. 5 Hippocampal ROS levels. Filled bars: control rats (C); open bars: irradiated rats (Rx). βE: 17-β-estradiol. $***p < 0.001$ respect to the respective control; §§§$p < 0.001$ respect to vehicle-injected, Rx rats. Data are mean of pmol DCF/mg tissue/min ± SEM.

behavioral radiation-induced changes. Hence, it will be suggested that βE could act through an antioxidant mechanism, without interacting with ERs. To confirm this hypothesis, an isomer known to hardly bind to ERs, αE (Yi *et al.* 2011), was administered to control and irradiated animals. The parameters that have been reversed by βE were assessed in αE-treated animals. Data of Fig. 6 show that treatment of irradiated rats with αE was effective in reversing the changes induced by neonatal ionizing radiation exposure. First, hippocampal ROS levels were increased in irradiated animals, whereas in αE-treated irradiated animals hippocampal ROS levels resulted significantly different from irradiated animals and similar to control values ($F_{1,31} = 9.717$, $p < 0.01$; C vs. Rx, $p < 0.001$; RxαE vs. Rx, $p < 0.001$, Fig. 6a). Second, αE-treated irradiated animals significantly decreased the number of lines crossed in the OF task in the second session, leading to habituation memory similar to control animals. However, the degree of significance was lower than that of control animals ($F_{1,57} = 3.466$, $p < 0.05$; first session vs. second session: C, $p < 0.001$; Rx, NS; RxαE, $p < 0.05$, Fig. 6b). Last, αE administered to irradiated animals was also effective in preventing radiation-induced effect on EPM performance ($F_{1,21} = 15.104$, $p < 0.001$; C vs. Rx, $p < 0.001$; RxαE vs. Rx, $p < 0.05$, Fig. 6c).

Discussion

Results show that administration of βE is effective in normalizing anxiety levels as well as in restoring the deficit in habituation memory in 30-day-old rats exposed to neonatal ionizing radiation. Moreover, while increased hippocampal ROS levels were found in irradiated rats, normal levels were observed in βE-treated irradiated rats. However, βE administration failed to prevent the increase in hippocampal total PKC activity observed in irradiated rats. Interestingly, the effectiveness of an isomer that is thought to hardly bind to ERs *in vivo*, αE, demonstrated that an antioxidant mechanism could underlie, at least partially, βE-mediated neuroprotection of several radiation-associated changes.

Several authors reported that αE is able to efficiently bind ERs *in vitro*, including a selective receptor ER-X (Toran-Allerand *et al.* 2005). In contrast, others stated that αE is an inactive isomer on ER when assessed *in vivo* (Yi *et al.* 2008), which suggest that the abundance of fatty acid in CNS might interfere with the binding of αE to ERs (Yi *et al.* 2011). Therefore, as the present experiments were made *in vivo*, it would be suggested that αE could poorly bind to ERs. In addition, since a re-establishment of hippocampal ROS was observed in either αE- or βE-treated irradiated animals, it could be proposed that neuroprotection would be mediated through an antioxidant mechanism (Kii *et al.* 2005; Prokai-Tatrai *et al.* 2008). However, it would not be discarded that both mechanisms, ER-dependent and independent, might act in concert.

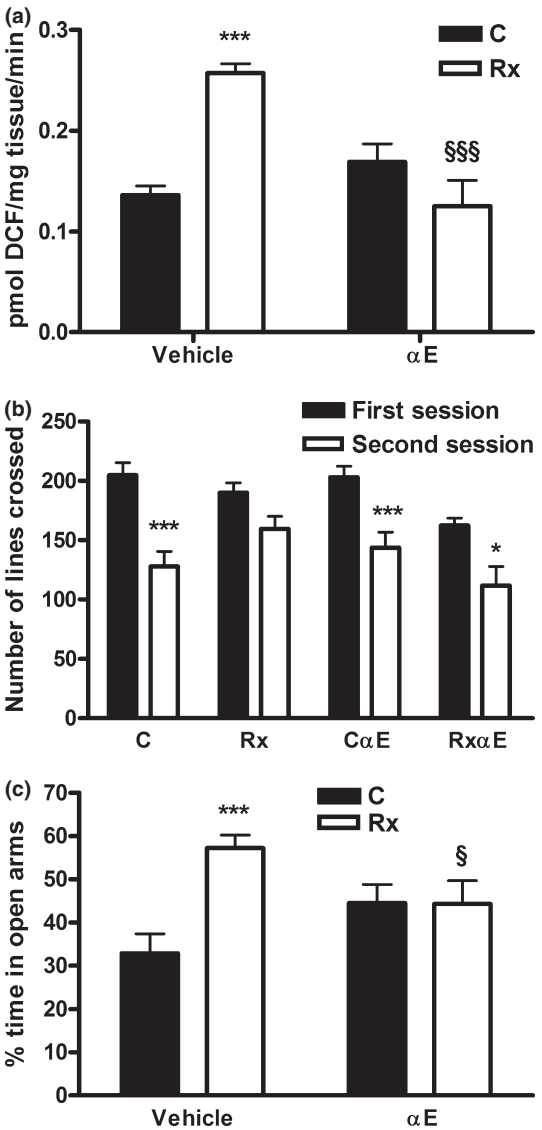

Fig. 6 Effect of αE on different behavioral and biochemical parameters. (a) Hippocampal ROS levels. Filled bars: control rats (C); open bars: irradiated rats (Rx). αE: 17-α-estradiol. ***$p < 0.001$ respect to the respective control; §§§$p < 0.001$ respect to vehicle-injected, Rx rats. Data are mean of pmol DCF/mg tissue/min ± SEM. (b) Number of lines crossed in the open field task. Filled bars: first session; open bars: second session. C: control rats; CαE: α-estradiol-treated control rats; Rx: irradiated rats; RxαE: α-estradiol-treated irradiated rats. *$p < 0.05$, ***$p < 0.001$ respect to the first session. Data are mean of the number of lines crossed ± SEM. (c) Percent time spent in open arms in the elevated plus maze task. Filled bars: control rats (C); open bars: irradiated rats (Rx). αE: 17-α-estradiol. ***$p < 0.001$ respect to respective control rats; §$p < 0.05$ respect to vehicle-injected, Rx rats. Data are mean of the percent of time spent in open arms respect to total time ± SEM.

As present data show that, in contrast to the reduced anxiety levels of irradiated rats, βE-treated irradiated animals have anxiety levels similar to control rats, it would be

suggested that βE might induce an increase in anxiety levels to achieve normal values in irradiated rats. This hypothesis supports Arabo *et al.* (2005) report, in which authors demonstrated that βE administered in the intrauterine stage is capable *per se* of increasing anxiety in adulthood. Interestingly, when estradiol was administered to adult female rodents, a decrease in anxiety was observed (Zhang *et al.* 2009; Walf and Frye 2010). However, in this work, in which developing males were treated with estradiol in the perinatal period and tested in adulthood, no changes in anxiety levels were observed when administered to control rats (Fig. 5), suggesting that this hormone would act within a window of opportunity by modifying differentially anxiety levels, depending on the developmental stage. In consequence, it could be suggested that an estrogen administered to neonatal animals has an organizing or differentiating action on brain that will produce a specific behavioral output in adulthood. Conversely, when administered in adulthood, the hormone might activate tissues organized previously in the development, resulting in adaptive changes that would involve a decrease in anxiety (Arnold 2009; Walf and Frye 2010). When a neuronal injury is interposed in neonatal animals, it seems that the estrogen attempts to re-establish normal brain organization, changing from an organizational to an activational role to reach normal anxiety levels. In other words, in the injured brain, estrogen administration might induce a recapitulation of certain developmental processes to protect, repair, and recover (Gillies and McArthur 2010).

Molecular mechanisms that could explain the neuroprotective action of estrogens include receptor-mediated genomic action (Sawada and Shimohama 2000; Zhao *et al.* 2004), receptor-mediated non-genomic action (Yu *et al.* 2004), and receptor-independent non-genomic action. The last relates to the antioxidant properties of their molecular structure (Mooradian 1993; Behl *et al.* 1997; Culmsee *et al.* 1999; Moosmann and Behl 1999; Daré *et al.* 2000; Prokai *et al.* 2003). ER-independent antioxidant effect seems to mainly depend on a non-specific mechanism of direct free-radical scavenging (Prokai-Tatrai and Prokai 2005), although indirect mechanisms, such as up-regulation of antioxidant enzymes (Garcia-Segura *et al.* 2001; Borrás *et al.* 2005), and chelation of redox-active metal ions (Ruiz-Larrea *et al.* 1995) would also be operating. In addition, a recent interesting publication shows that the lipophilicity of estratrienes leads to their accumulation in the hydrophobic plasma membranes affecting membrane fluidity, placing the steroid at the site of peroxidation and preventing oxidative damage (Yi *et al.* 2011). Moreover, an additional antioxidant mechanism was recently reported by Borrás *et al.* (2010), who postulate that stimulation of mitochondrial ERs would mediate induction of antioxidant enzymes *in vitro*. However, whether this pathway is necessary and sufficient to mediate the neuroprotective effects of estrogens *in vivo* still remains to be elucidated.

Present results show that βE was effective in preventing hippocampal ROS increase induced by neonatal ionizing radiation exposure, supporting an antioxidant mechanism for βE (Prokai-Tatrai *et al.* 2008). To confirm this hypothesis, αE, an optical isomer of βE that is more than 40-fold less potent on ERs and a naturally occurring compound in the pregnancy (derived from epitestosterone), was used. Although with lower statistical significance than βE, αE has proven to be effective in most parameters measured. Therefore, taking into account (i) the weak action of αE on ERs; (ii) that α- and βE have proved to have similar neuroprotective effects; and (iii) that ROS levels were efficiently restored after steroid treatments, it would be suggested that βE may act, at least in part, through an antioxidant mechanism ER-independent in the present irradiation model, supporting Prokai and Simpkins (2007), McClean and Nuñez (2008) and Yi *et al.* (2008) results. However, it would not be discarded that other signaling mechanisms, including genomic and non-genomic actions mediated by ERs, may underlie βE neuroprotection (Garcia-Segura *et al.* 2001; Toran-Allerand *et al.* 2005; Prokai and Simpkins 2007; Morissette *et al.* 2008). With the plethora of potentially neuroprotective pathways activated by estrogens, it is unlikely that a single mechanism is responsible for their neuroprotective action. Therefore, a possible involvement of ER in the neuroprotective actions of βE cannot be excluded.

The literature shows some discrepancies concerning the optimal dose of estradiol needed to obtain a neuroprotective effect, both *in vitro* and *in vivo*. This may be due, in part, to the different hormonal concentration requirements of different brain areas at different developmental ages. In fact, it is noteworthy that in some studies *high* estradiol concentrations were used to achieve neuroprotection through an antioxidant mechanism *in vitro* (Behl *et al.* 1997; Vedder *et al.* 1999) and others use *moderate* concentrations of βE *in vitro* (1 μM) that demonstrated to be neuroprotective through an antioxidant mechanism (Culmsee *et al.* 1999; Zorrilla Zubilete *et al.* 2011). On the other hand, authors such as Prokai *et al.* (2003) showed that *moderate* levels of estradiol can be neuroprotective acting through an antioxidant mechanism *in vivo*. Interestingly, neuroprotective properties of estrogens independent from ER have been observed also in studies made *in vivo* using physiological concentrations of estrogen analogs that do not bind ER (Liu *et al.* 2002). Under our experimental conditions, pharmacological (above the normal physiological range) serum levels were found 30 min after the first administration of 50 μg/kg of βE (1975 ± 22 pg/mL over basal values). Therefore, this concentration was present at the moment of the radiation exposure and is comparable with serum levels found in estradiol-treated animals subjected to ischemic injury (Yang *et al.* 2000; Sandstrom and Rowan 2007) or in intact animals (MacLusky *et al.* 2005). Moreover, the finding of a normalization of ROS levels in irradiated hippocampi suggests that it is possible that high

estrogen concentrations obtained *in vivo* could mediate a direct scavenging activity. It is possible that the pharmacological levels of estradiol that remained elevated at the time of radiation exposure would be responsible for the neuroprotective findings reported here. However, the severity of the lesion and the route of administration may also be important determinants of the hormone concentration required to counterbalance the damage.

The lack of efficacy of βE on the persistent increase in total PKC activity might be probably because of the differential regulation of PKC isoforms. Preliminary data show that an increase in PKCβ1 levels, an isoform known to be up-regulated by ROS (Alzamora and Harvey 2008; Almeida *et al.* 2010), was induced in the Hip of irradiated animals, whereas normal levels were observed in the Hip of βE-treated irradiated animals (data not shown). Therefore, it would be suggested that βE will probably affect differentially PKC isoenzymes, by increasing some and decreasing others, resulting in unchanged total PKC activity. Future studies are required to confirm this hypothesis.

On the other hand, while βE administration was ineffective in modifying the altered IA task performance in irradiated animals, the inability of irradiated rats to habituate to the environment in the OF (Caceres *et al.* 2010) was counteracted in rats treated with βE. These opposite results might be related to the multiple signaling pathways involved in the consolidation of habituation memory measured in the OF in contrast to the few needed in an aversive task such as IA. Specifically, interfering with one of the multiple pathways involved in OF habituation memory may be enough to counteract habituation memory impairment, while in a stressful task is more difficult to counteract the change induced by an exogenous agent (Vianna *et al.* 2001). Finally, it should not be overlooked that βE may have variable effects on learning and memory in animal models, which may be a result of differential effects of the drug at training and/or testing, depending on the task used (Rhodes and Frye 2006).

The non-significant radiation-induced decrease in granule cells number found in DG of irradiated Hip (Caceres *et al.* 2010) could be attributed to a decrease in cell proliferation, a process that could be linked to learning and memory mechanisms, as reported by Hellström *et al.* (2009) in adult irradiated animals. Moreover, considering the promoting effect of estradiol on proliferation (Galea 2008), it would be hypothesized that in irradiated animals βE would counteract the potential decrease in proliferation, raising the proliferative cell number to control values and contributing to the re-establishment of behavioral abnormalities. In consequence, the number of proliferative cells in hippocampal DG was estimated using doublecortin, a marker of precursor neurons. Results show that in 30-days-old Hip, doublecortin immunoreactive neuronal precursors with well-developed processes were not detected and the staining was dispersed, as reported by Hwang *et al.* (2008). When proliferation was quantified, although a decrease in the proliferative cells number in irradiated Hip was observed, no statistical significance was found when compared with controls. Moreover, when βE was administered to control and irradiated rats, no changes in proliferation were produced in proliferative cells number, suggesting that βE was unable to affect proliferation in our model (see Appendix S1). Therefore, it would be postulated that radiation-induced behavioral changes would not be caused by alterations in cell proliferation.

Because of differences between species, we had to inject the rat pups on postnatal life to have a model relevant for understanding injury in humans, since the developmental stage of a neonatal rat correlates with this of human premature baby. In consequence, it is recommended that care should be taken to avoid radiation exposure of the fetus and, in particular, upon the exposure of prematurely born children to different environmental agents (Altman 1987). Although therapeutic irradiation is rarely used in these patients, it would not be discarded that an accidental irradiation of a pregnant woman or a premature baby could occur. Interestingly, these alterations might be prevented by an early βE treatment.

In conclusion, our results support the hypothesis that βE might disrupt the mechanism of radiation-induced ROS generation, leading to the re-establishment of the behavioral changes triggered by ionizing radiations suggesting that βE would be acting mainly through an antioxidant mechanism. The finding of neuroprotection mediated by αE reinforces this hypothesis. Therefore, we reasoned that a substantial portion of the neuroprotective activity of estrogens was ER-independent.

Finally, since estrogens are widely used therapeutic compounds with a thoroughly evaluated safety profile and in view of the excellent safety history of estrogen therapy, there is sufficient evidence to support the subchronic use of estrogen as preventive tools after different CNS insults.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Supplemental data.

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from

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