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Pharmacological alterations that could underlie radiation-induced changes in associative memory and anxiety

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ABSTRACT

It is widely known that ionizing radiation is a physical agent broadly used to kill tumor cells during human cancer therapy. Unfortunately, adjacent normal tissues can concurrently undergo undesirable cell injury. Previous data of our laboratory demonstrated that exposure of developing rats to ionizing radiations induced a variety of behavioral differences respect to controls, including changes in associative memory and in anxiety state. However, there is a lack of data concerning modifications in different related pharmacological intermediaries.
Therefore, the aim of the present study was to investigate whether the behavioral differences observed in young animals irradiated at birth might be underlain by early changes in PKCß1 levels which, in turn, could lead to changes in hippocampal GABAergic neurotransmission.
Male Wistar rats were irradiated with 5 Gy of X rays between 24 and 48 h after birth. Different pharmacological markers related to the affected behavioral tasks were assessed in control and irradiated hippocampus at 15 and 30 days, namely $GABA_A$ receptor, $GAD_{65–67}$, ROS and PKCß1.
Results showed that all measured parameters were increased in the hippocampus of 30-days-old irradiated animals. In contrast, in the hippocampus of 15-days-old irradiated animals only the levels of PKCß1 were decreased. These data suggest that PKCß1 might constitute a primary target for neonatal radiation damage on the hippocampus. Therefore, it could be hypothesized that an initial decrease in the levels of this protein can trigger a subsequent compensatory increase that, in turn, could be responsible for the plethora of biochemical changes that might underlie the previously observed behavioral alterations.

1. Introduction

It is widely known that ionizing radiation is a physical agent broadly used to kill tumor cells during human cancer therapy. Unfortunately, adjacent normal tissues can concurrently undergo undesirable cell injury. In particular, harmful radiation-induced effects on healthy Central Nervous System (CNS) have been observed. Of relevance, CNS lesions in children exposed to radiation at the time of Hiroshima and Nagasaki atomic explosions as well as over the course of the Chernobyl and Fukushima accidents have been reported (UNSCEAR, 1993; Kimler, 1998; Fushiki, 2013; Palgi et al., 2012).

Previous data of our laboratory demonstrated that exposure of developing rats to ionizing radiations induced a variety of behavioral differences when compared to controls, including changes in associative memory and in anxiety state (Caceres et al., 2010, 2011, see a summary of these results in Table 1). We have proposed that the improvement in the performance in an associative memory task detected in irradiated (Rx) neonatal animals could be underlain by a lower level of emotional reactivity, featured in these animals as decreased anxiety levels. It should be highlighted that some authors have hypothesized that an anxious behavior might interfere with the learning of a specific task (Martín-García et al., 2008), supporting our behavioral results.

The hippocampus (HC) is a well-known brain structure involved in learning and memory processes. In particular, the inhibitory avoidance task (IA), which is used to evaluate associative memory skills, has been shown to depend on the HC (Vianna et al., 2000a). It is one of the most highly connected areas of the brain and, although has traditionally been considered the "memory area", it started to emerge as a brain integrator of emotion and cognition (Femenía et al., 2012). Actually, it has been postulated that HC can interact with other brain structures – such as the amygdala – to form emotional memories (Phelps, 2004). In fact, the involvement of HC in the development of anxiety behavior has been demonstrated by lesion and pharmacological studies (Fuss et al., 2010).

Therefore, as changes in associative memory and anxiety levels were found in neonatally Rx animals and given that hippocampal histology

Abbreviations: ROS, reactive oxygen species; HC, hippocampus; CNS, central nervous system; Rx, irradiated; Ct, control; IA, inhibitory avoidance; OF, open field; EPM, elevated plus maze; PK, protein kinases; GAD, glutamic acid decarboxylase; PND, postnatal day; OD, optical density.

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Table 1
Behavioral data obtained previously by[(1)] Caceres et al. (2010) and[(2)] Caceres et al. (2011), with editorial permission.

Treatment group	Behavioral task	
	Associative memory (T2/T1)	Anxiety levels (% time in open arms)
Ct	24.74 ± 5.7 [(1)]	36.66 ± 5.4 [(2)]
Rx	51.62 ± 8.2 * [(1)]	57.21 ± 2.9 ** [(2)]

Associative memory: Data are mean of the latency to enter the dark compartment in the inhibitory avoidance task (ratio T2/T1) ± SEM. [t = 2.7; df = 17; * = $p < 0.05$ respect to control].
Anxiety levels: Data are mean of the percent of time spent in open arms respect to total time ± SEM in the elevated plus maze task. [t = 3.4; df = 13; ** = $p < 0.01$ respect to control]. All data were obtained from postnatal day 30 (PND 30) animals.
Ct: Control animals; Rx: Irradiated animals.

was also affected (Caceres et al., 2010), it could be postulated that a number of behavior-related HC pharmacological markers may be disturbed.

Indeed, protein kinases (PK) have long been known to be involved in different HC-related memory processes. In particular, PKC comprises a family of isoenzymes that can be activated by rises in intracellular calcium and lipid mediators and has been extensively implicated in pre- and postsynaptic events related to neuronal plasticity (Majewski and Iannazzo, 1998; Vianna et al., 2000b). PKC family includes the mostly postsynaptic isoforms PKCα and ß and the pre and post-synaptic isoform PKCγ (Wu et al., 2007). Since PKCß1 is believed to be one of the behaviorally relevant PKC isoforms that were shown to be involved in the early synaptic events responsible for the acquisition and consolidation of IA learning, mainly in short-term memory (Paratcha et al., 2000), it could be suggested that this isoform could be involved in the changes observed in rats exposed to ionizing radiation at birth.

Actually, total PKC activity was increased in the HC of 30-days-old Rx rats, together with reactive oxygen species (ROS) levels (Caceres et al., 2010). Interestingly, it has been reported that there seems to be an interactive and feed-forward relationship between radiation-induced ROS and PKC, whereby ROS production can induce an increase in PKC activity and its downstream pathways and PKC can also activate ROS production and related downstream pathways (Inoguchi et al., 2000; Dai et al., 2006; Caceres et al., 2010). Therefore, it could be suggested that the observed HC-related behavioral changes induced by neonatal ionizing radiation exposure could be triggered by changes in the levels of any isoform of hippocampal PKC. Moreover, as PKCß1 is known to be up-regulated by ROS, one of the key mediators of radiation-induced damage (Guelman et al., 2003; Alzamora and Harvey, 2008; Almeida et al., 2010; Caceres et al., 2010), we presumed that its expression could be increased in the irradiated HC and that radiation exposure in early developmental stages might also interfere with translocation/activation of this isoform during the learning procedure.

On the other hand, it is known that anxiety behavior is mainly mediated by $GABA_A$ receptors ($GABA_A$Rs), as alterations in benzodiazepine and $GABA_A$Rs binding sites have been associated with altered anxiety-related behavior in both humans and rodents (Barros et al., 2006). GABA neurotransmitter and its receptors are critical for the development and coordination of neuronal activity and are essential for most of the physiological and behavioral processes in the brain, including memory and anxiety behaviors. Therefore, it could be suggested that the decrease in anxiety levels observed in Rx rats might be related to an over-stimulation of the GABAergic neurotransmission.

$GABA_A$ receptors belong to a large and diverse family of Cl^--permeable ion channels that mediate fast transmission at inhibitory GABAergic synapses, reducing excitability (Cid et al., 2011) by inhibiting the positive-feedback loop that underlie normal nervous system development and/or excitotoxicity (Zhang et al., 2007). In the HC, abundance of $GABA_A$Rs is particularly high in the CA1 and CA3 regions and in the subiculum. In addition, it has long been established that a direct relationship exists between the number of synaptic $GABA_A$Rs at the cell surface and the strength of inhibition at the synapse (Abramian et al., 2010).

Certain disorders are thought to be associated with low anxiety levels, together with $GABA_A$R hyperfunction. Interestingly, recent studies using an animal model of human Down syndrome have shown the reversal of the syndrome's associated behavioral defects using a $GABA_A$R antagonist (Hines et al., 2012). Conversely, a deficit in GABAergic inhibitory control is one of the major hypotheses underlying the symptomatology of schizophrenia (Lewis et al., 2005; Benes et al., 2007). Interestingly, pathology of GABAergic neurons is usually accompanied by a compensatory upregulation of $GABA_A$R on target neurons (Biggio et al., 2007). Thus, enhancing GABAergic transmission through and increase in $GABA_A$R would be expected to rectify at least some of the pathophysiological deficits. Alternatively, an augmentation in $GABA_A$R density triggered under a pathological condition might itself induce an increase in GABA neurotransmitter to avoid inhibitory imbalance and to help reach a more balanced state. Therefore, measurement in our irradiation model of the levels of glutamic acid decarboxylase (GAD), the enzyme that converts glutamate to GABA, could shed light about these different hypotheses because GAD expression is closely related to GABAergic activity (Kash et al., 1999).

Finally, given that experimental data demonstrated that $GABA_A$Rs can directly associate with the isoform PKCß in neurons (Brandon et al., 1999, 2000, 2002), it could be suggested that increased hippocampal PKCß1 levels might underlie an increased $GABA_A$R density and enhancement of GABAergic neurotransmission.

Then, the aim of the present study was to investigate whether the previous behavioral alterations observed in young animals irradiated at birth might be underlain by early changes in PKCß1 levels which, in turn, could lead to changes in hippocampal GABAergic neurotransmission. In addition, the time course of the appearance of the observed modifications in the irradiated HC was determined to establish a temporal relationship.

2. Materials and methods

2.1. Animals

Healthy male and female albino Wistar rats were obtained from the Animal Facilities of the Biochemistry and Pharmacy School of the University of Buenos Aires, Argentina.

A total of 10 cages with one male and two females each (total females $n = 20$; total males $n = 10$) were used in the present study for mating procedures. When pregnancy of each female became evident (*e.g.*, few days before delivery), pregnant rats were isolated one per cage and left undisturbed until delivery.

The day of birth was designated as PND 0 and was determined by the inspection of the cages three times per day. Only male rats, coming from twenty litters, were used for the different experimental procedures. PND 1 animals were randomly assigned to each experimental group: control (Ct) or irradiated (Rx).

A subset of rats ($n = 42$) was exposed to ionizing radiation and another subset – the Ct, sham-exposed rats ($n = 42$) – was placed in the same device than radiation-exposed rats, but without being subjected to the procedure of irradiation.

All groups were kept with their dams until PND 21, with the exemption of those used at PND 15. Thereafter, they were separated and maintained 3–4 per cage until PND 30, with food and water *ad libitum*, on 12 h light–dark cycles (lights on at 7 A.M.) at 22 ± 2 °C with wood shavings for bedding.

At PND 15 or 30, only the males were randomly assigned to a different parameter evaluation for each treatment group. Then, the 42 animals of each group were randomly assigned to each of the following groups: a) ROS measurement; b) Binding to $GABA_A$ receptor; c) protein levels of PKCß1 and GAD_{65-67}. All experiments were done in PND 15 and

30 rats and the number of animals in each experimental group was $n = 7$, for a done endpoint (total animals used: 84).

Animals were handled and sacrificed 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 503/10. The CICUAL adheres to the rules of the "Guide for the Care and Use of Laboratory Animals" (NIH) (2011 revision) and to the EC Directive 86/609/EEC (2010 revision) for animal experiments. Adequate measures were taken to reduce the number of animals used and to minimize their pain or discomfort.

To avoid circadian rhythm alterations, radiation exposures were conducted in the late phase of the light cycle, between 4 P.M. and 6 P.M. At PND 15 or 30, the experiments were performed.

2.2. Irradiation procedure

Only the heads of neonatal male Wistar rats, of ages 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, 6 MV of photon energy). The dose-rate was of approximately 1 Gy/min, being the total time of radiation exposure approximately 5 min. Radiation energy absorbed into the tissue was approximately 5 J/kg, since 1Gy 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, as at the dose of radiation used in the present study, no animal deaths were produced. All radiation safety standards were followed.

2.3. ROS determination

The levels of hippocampal ROS were determined according to the method described by Driver et al. (2000). Briefly, HC were homogenized in ice Locke's solution (0.5 mg of tissue/ml). Aliquots of the homogenate were taken and left to warm at room temperature during 5 min. 10 μl of dichloro-fluorescein diacetate (0.97 mg/ml in methanol) was added (10 μM final concentration) and the mixture was incubated at room temperature during 15 min. Finally, the fluorescence was measured at 485 nm (excitation) and 530 nm (emission). A standard curve was performed using oxidized dichloro-fluorescein (DCF). Results were calculated as pmol DCF/mg tissue/min and expressed as mean values ± SEM.

2.4. $GABA_AR$ binding

2.4.1. Preparation of hippocampal synaptosomes

The HC was isolated from Ct and Rx animals and synaptosomes were purified on discontinuous Percoll gradients as described previously (Cid et al., 2008). These nerve terminals are subcellular membranous structures that are formed during the mild disruption of the brain tissue and retain the morphological features and chemical composition of the presynapses. The synaptosome is the simplest preparation that possesses all machinery for synaptic vesicle exocytosis and endocytosis and contains cytoskeleton and organelles in addition to synaptic vesicles with neurotransmitters and receptors for reuptake at the presynaptic zone. Furthermore, synaptic $GABA_ARs$ are mainly localized in the postsynapsis of synaptosomes which is a small bit of the neuronal soma (Cid et al., 2007).

Synaptosomes which sedimented between the 10 and 23% Percoll bands were collected and diluted in a final volume of 30 ml of HEPES buffer medium consisting of 140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1 mM $MgCl_2$, 1.2 mM Na_2HPO_4, 10 mM glucose, and 10 mM HEPES, pH 7.4, before centrifugation at 27,000 × g for 10 min at 4 °C. The pellets thus formed were resuspended in 5 ml of HEPES buffer medium, and the protein content was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA). After determination of total protein, [^{3}H]-flunitrazepam binding assay was performed.

2.4.2. [^{3}H]-flunitrazepam binding assay

The specific binding of [^{3}H]-flunitrazepam (85 Ci/mmol) was measured by a filtration technique (Cid et al., 2008). Binding was carried out in the presence of radioligand at final concentrations of 0.5, 1, 2, 3, 4, 5, 8, and 9 nM, at 4 °C. Each assay was performed in triplicate using 1-ml aliquots containing 0.3 mg of proteins from the synaptosomal fractions. Nonspecific binding was measured in the presence of 10 μM diazepam. After 60 min of incubation, samples were filtered under vacuum through Whatman GF/B filters using a Brandel M-24 filtering manifold. Samples were washed three times with 4 ml of ice-cold Tris–HCl buffer (50 mM, pH 7.4) and the radioactivity was measured using an LKB-1214-RackBeta counter at 60% efficiency. The values Kd and Bmax were obtained by nonlinear regression using the equation for hyperbola (one binding site): $Y = Bmax*X/(Kd + X)$, where Bmax is the maximal binding, and Kd is the concentration of ligand required to reach half-maximal binding. Results are expressed as % of respective controls ± SEM.

The central benzodiazepine receptor density can be used to express the $GABA_A$ receptor density, because the flunitrazepam-binding site is located in the α-subunit of the $GABA_A$ receptor.

2.5. Western blot assay

The hippocampi were homogenized by sonication in lysis buffer (1:2 w/v) containing DTT 1 mM, PMSF 0.2 mM, Leupeptine 1 μM, Octylphenyl-polyethylene glycol (Igepal) 0.5%. The homogenates were centrifuged at 10,000 *g* for 5 min at 4 °C. An aliquot from the supernatant was taken for protein determination by Bradford technique. For western blot analysis, lysate aliquots containing 50 μg of protein were denatured by the addition of loading buffer (Tris–HCl 50 mM pH 6.8, SDS 1%, glycerol 10%, bromophenol blue 0.05% y 2-β-mercaptoethanol 140 mM) and subsequent boiling for 3 min.

The samples were then separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, UK) using a Bio-Rad Transblot Mini II (100 V constant voltage for 1 h at 4 °C). Proteins on PVDF membranes were visualized by reversible staining with Ponceau-S solution (Sigma, Co., MO, USA) and washed in Tris-buffered saline (TBS).

Membranes were blocked at room temperature in milk buffer (5% non fat dry milk in TBS Tween 0.1%) and then incubated overnight at 4 °C with anti-$GAD_{65–67}$ (1:1000, Abcam) or anti-PKCß1 (1:250, Abcam) polyclonal antibodies overnight at 4 °C. Subsequently, membranes were incubated for 2 h at room temperature with 1:5000 anti-mouse IgG–horseradish peroxidase-conjugated secondary antibodies (Abcam Inc.). After washing with TBS Tween 0.1%, membranes were analyzed by enhanced chemiluminescence (Amersham Biosciences, UK). The optical density (OD) of the bands on films was determined by quantitative densitometry with a computerized image processing system (GE healthcare ImageQuant) and the analysis of the data was made through the software Image J (v 1.43u, National Institutes of Health, Bethesda, MD, USA). After stripping, the membranes were probed with anti ß-actin antibody, to normalize the results. Optic density (OD) values for each band were relativized to the OD values of ß-actin for each lane.

2.6. Statistical analysis

Significant differences between groups were analyzed using student *t* test (SigmaStat, v. 3.5). Results are expressed as mean values ± SEM. A probability <0.05 was accepted as significant. All results fitted normal parametric data.

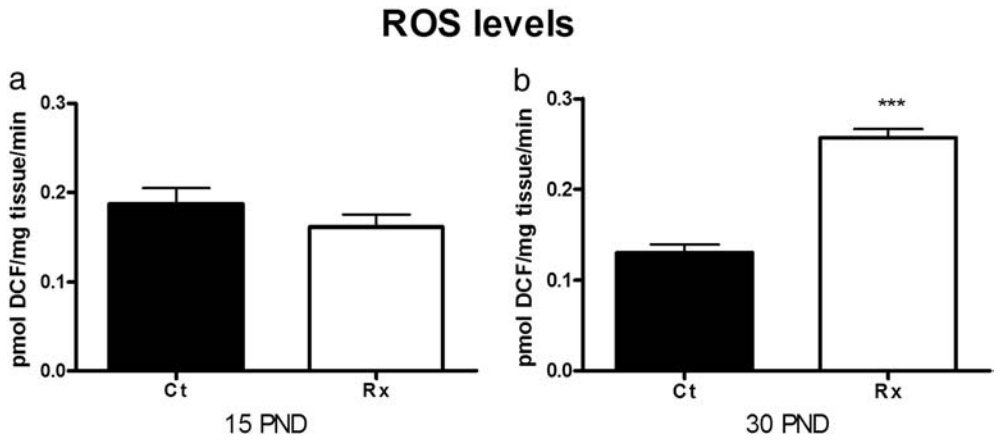

Fig. 1. Hippocampal ROS levels increased in 30-days-old animals. Filled bars: Ct (control rats); open bars: Rx (Irradiated rats). (a) No changes in ROS levels were observed in irradiated 15-days-old animals; (b) a significant increase in ROS levels was found in 30-days-old animals. *** $p < 0.001$ respect to Ct. Data are mean of the concentration of pmol DCF/mg tissue/min ± SEM. $n = 7$ rats per group.

3. Results

3.1. Changes in associative memory and anxiety levels were found in irradiated PND 30 animals

Data of previous studies performed in our laboratory show significant changes in irradiated PND 30 animals when different parameters were measured through anxiety and associative memory tasks (Table 1).

3.2. ROS levels increased in the HC of irradiated PND 30 animals, without changes at 15 days

Data show that 15 days-old irradiated HC have ROS levels similar to the respective controls (Fig. 1a). However, a significant increase was observed at 30 days (Fig. 1b).

3.3. Hippocampal PKCß1 levels decreased at PND 15 and increases at PND 30

In contrast, a decrease in PKCß1 levels was found in HC of irradiated 15-days-old rats when compared with the respective controls (Fig. 2a) whereas at 30 days a significant increase was found (Fig. 2b).

3.4. Hippocampal $GABA_AR$ binding increased in 30-days-old irradiated animals

Student *t* test revealed that Bmax value of Rx animals resulted similar to Ct at 15 days, whereas a significant effect of ionizing radiation on Bmax values was found in 30-days-old animals (Fig. 3a), being the Bmax value found in synaptosomes from Rx rats significantly higher than Ct in PND 30 rats (117.7 ± 6.5% of the Ct, $p < 0.05$). Similarly, although Kd was unchanged in irradiated 15-days-old HC, a significant increase was observed at 30 days (184.3 ± 28% of the Ct, $p < 0.01$, Fig. 3b).

3.5. GAD_{65-67} levels increased in the HC of 30-days-old irradiated animals

Data show that whereas hippocampal GAD_{65-67} levels of Rx animals did not differ from Ct at 15 days post-irradiation, a significant increase was observed at 30 days (Fig. 4a and b).

4. Discussion

Results showed that neonatal ionizing radiation exposure induced an initial decrease in hippocampal PKCß1 levels, observed as early as 15 days after exposure, followed by a rise in PKCß1 and ROS levels at 30 days. In addition, concurrent increases in $GABA_AR$ binding

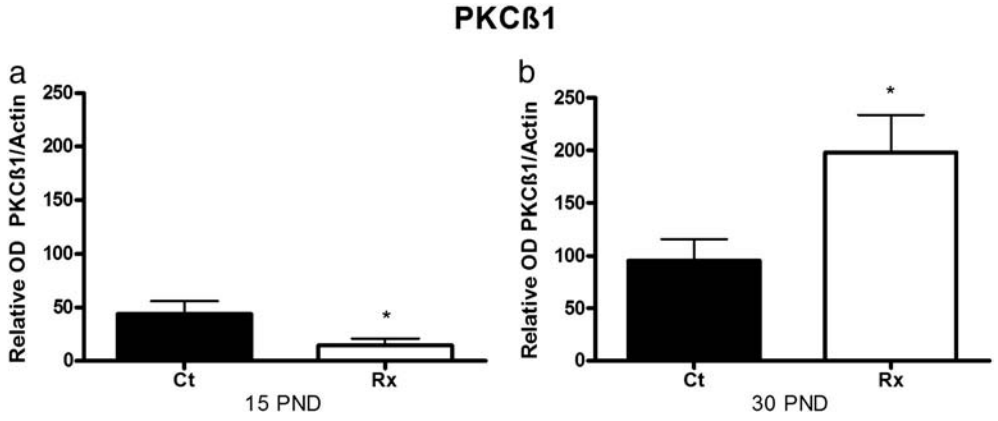

Fig. 2. Hippocampal PKCß1 levels decreased in 15-days-old animals and increased in 30-days-old animals. Filled bars: Ct (control rats); open bars: Rx (Irradiated rats). (a) A decrease in hippocampal PKCß1 levels was observed in irradiated 15-days-old animals; (b) a significant increase in PKCß1 levels was found in 30-days-old animals. * $p < 0.05$ respect to Ct. Results are expressed as the ratio of the optical density (OD) of the PKCß1 band and the ß-actin. Values are expressed as means ± S.E.M. $n = 7$ rats per group.

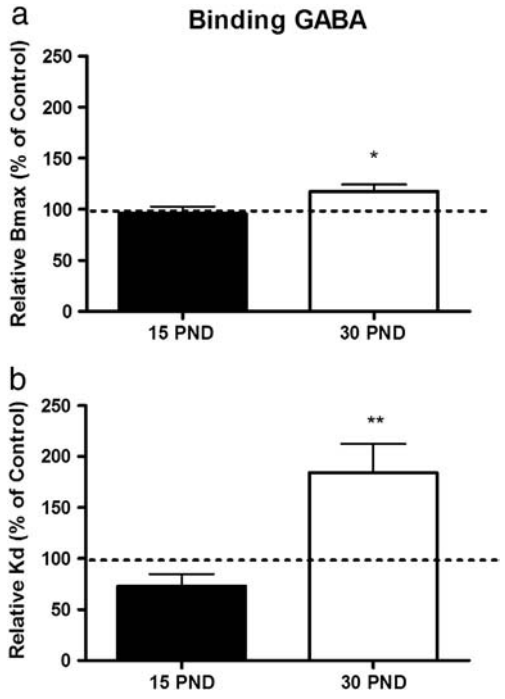

Fig. 3. Hippocampal $GABA_AR$ binding increased in 30-days-old irradiated animals. Filled bars: Ct (control rats); open bars: Rx (Irradiated rats). (a) No changes in Bmax values were observed in irradiated 15-days-old Rx animals, whereas significant changes were induced in PND 30 animals; (b) Whereas no changes were induced in the Kd of PND 15 animals, a significant increase was found in PND 30 animals. *, **, $p < 0.05$ and 0.01 respect to Ct. Data are mean of: (a) Bmax and (b) Kd expressed as % of control (·····). $n = 7$ rats per group.

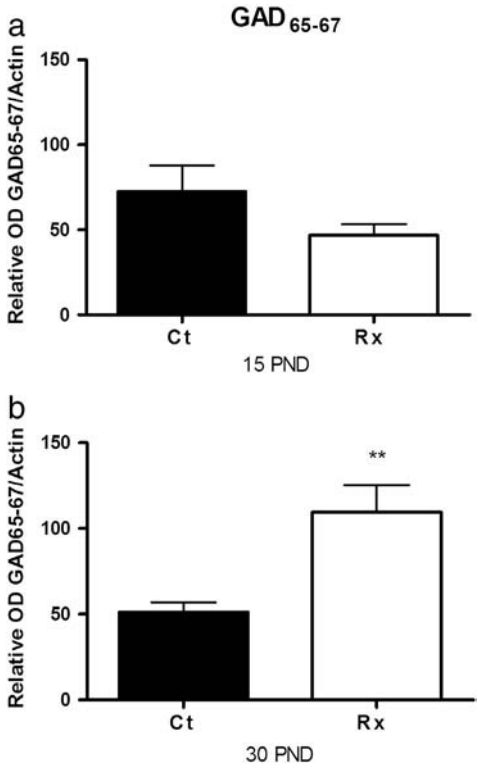

Fig. 4. $GAD_{65–67}$ levels increased in the HC of 30-days-old irradiated animals. Filled bars: Ct (control rats); open bars: Rx (Irradiated rats). (a) No changes in $GAD_{65–67}$ levels were observed in irradiated 15-days-old animals; (b) a significant increase in $GAD_{65–67}$ levels was found in 30-days-old animals. ** $p < 0.01$ respect to Ct. Results are expressed as the ratio of the optical density (OD) of the $GAD_{65–67}$ band and the ß-actin. Values are expressed as means ± S.E.M. $n = 7$ rats per group.

parameters and in GAD levels were observed in the hippocampus of irradiated 30-days-old animals, without changes at 15 days. These findings suggest that the behavioral differences observed previously in 30-days-old animals irradiated at birth might be underlain by specific pharmacological alterations.

It is well known that PKC plays a fundamental role in the regulation of neuronal survival, death, and differentiation (Maher, 2001). Its activation might be involved not only in neuroprotection mechanisms but also in the neuropathology of several neurodegenerative diseases (Tomimatsu and Arakawa, 2008). Different authors found that an increase in PKC levels would correlate with a better performance in different learning and memory tasks. Cammarota et al. (1997) reported that the translocation of PKC from cytosol to membrane was produced after LTP and IA learning in chicks and rats. In particular, Wu et al. (2007) demonstrated that PKCß1 up-regulation could be, at least in part, responsible for enhanced learning and memory in a model of prenatally stressed offspring. On the other side, it has been reported that prenatal exposure to a psychotropic drug such as heroin impaired memory and blocked the translocation of PKCß1 in the HC of mice (Huleihel and Yanai, 2006). Since PKC levels – in particular PKCß1 – were increased in 30-days-old HC of irradiated rats and given that a better performance in IA task was observed in these animals (Caceres et al., 2010), the hypothesis that PKCß1 changes might affect associative memory is supported by present results.

The simultaneous increases in PKCß1 and ROS levels observed in 30-days-old irradiated HC reinforce the key role that ROS play in the activation of PKC, mainly PKCß1, supporting the interactive and feed-forward relationship between both molecules as reported previously (Inoguchi et al., 2000; Dai et al., 2006; Adiga and Nair, 2008; Caceres et al., 2010).

The finding of an initial decrease in hippocampal PKCß1 levels (e.g., at 15 PND) without parallel changes in ROS levels suggested that ionizing radiation exposure could be inducing a deficit of PKCß1 in developing animals, probably acting through a mechanism not dependent from the formation of ROS. Furthermore, a subsequent compensatory PKCß1 upregulation would be triggered in order to counteract potential damage that could result from this imbalance. Future experiments will be done to confirm this hypothesis.

On the other hand, present data suggest that the increase in hippocampal $GABA_AR$ receptor density and affinity in irradiated PND 30 rats could have a causal connection with the previously found decrease in anxiety levels which probably might, in turn, underlie associative memory changes. Interestingly, Martín-García et al. (2008) suggested that memory improvement goes together with anxiolysis, since they found that the drug finasteride deteriorated passive avoidance behavior in adulthood and induced an anxiogenic-like profile. Moreover, other evidences indicated that HC might also have a role in anxiety and emotional behaviors in addition to its role in learning and memory (Bannerman et al., 2004). Therefore, the examination of hippocampal GABAergic neurotransmission could be relevant for the understanding of our previous behavioral findings.

Although the increase in $GABA_AR$ density and affinity observed in irradiated HC might arise from a more primary deficit in GABA, as a way of compensating its deficiency, the enhanced hippocampal $GAD_{65–67}$ levels found in irradiated animals allows us to infer that GABA levels could be also increased and demonstrates that the GABAergic system can be able to compensate for detrimental radiation-induced changes, acquiring a new inhibitory status.

Actually, the differences observed in the flunitrazepam-sensitive $GABA_AR$ binding observed in irradiated rats' hippocampi suggest that basal and/or compensatory changes in the GABAergic system could be involved in the pathophysiological mechanism of ionizing radiation in developing animals. It could be hypothesized that the elevated inhibition in the irradiated HC may antagonize some radiation-induced pharmacological changes as postulated by Clarkson et al. (2010) in a model of stroke, resulting "excessively" inhibited, underlying the observed

decrease in anxiety levels. Further experiments should be made to confirm this hypothesis.

Importantly, neural circuits of immature brain in developing rats are still excitatory dominant until approximately postnatal day 21–22, due to the late development of inhibitory circuits. Therefore, it should not be discarded that the increase in $GABA_AR$ density and affinity, as well as the increase in GAD levels, observed in PND 30 irradiated animals could counteract an initially exacerbated excitotoxic synaptogenesis that might be induced after radiation exposure (Depino et al., 2008). Therefore, the changes reported here could emerge as a consequence of a late compensatory response of the GABAergic system to the noxious agent, leading to an increased behavioral inhibition, instead of being a primary alteration of ionizing radiation on GABAergic function. Since GABA homeostasis during development contributes to setting the levels of anxiety-related behaviors in adulthood, it could be proposed that the excessive number of GABA receptors observed in the HC of irradiated animals might fail in establishing "normal" levels of anxiety.

The enhanced anxiolytic action induced by ionizing radiation exposure may be related to an increased GABA release mediated through elevated GAD (Tien-Hsiung et al., 2011). Therefore, the increased expression of GAD highly suggests that presynaptic GABA synthesis might be increased after irradiation and that modulation of GAD could underlie anxiety-like behaviors, supporting data of Kash et al. (1999) which found that GAD_{65} deficient mice exhibit increased anxiety-like behaviors in the open field and elevated zero maze tests.

Essentially, present experiments point out that some changes induced in the neonatal period may have behavioral consequences in adolescence: the reaction to emotional cues (elevated plus maze (EPM) task to test anxiety) and the capability to learn experiences associated with high emotional stimuli as punishment (IA task to test associative memory). Thus, it could be suggested that the stress induced by a big stressor, an electric foot-shock punishment, at the end of the training session of the IA task could interact with the putative "anxiolytic effect" of ionizing radiation to produce a better performance.

Little is known about the endogenous mechanism by which neurons control the functional properties of $GABA_AR$ subtypes that mediate inhibition. One way in which modulation occurs is via posttranslational modifications of the synaptic $GABA_AR$. Specifically, the phosphorylation of key residues on synaptic $GABA_AR$ subunits regulates the extent to which the $GABA_AR$ will interact with protein complexes responsible for endocytosis from and insertion to the cell membrane (Abramian et al., 2010). It has been reported that PKC can increase both the phosphorylation and cell surface stability of the α4 subunit of $GABA_AR$ in hippocampal slices, a phenomenon that could be correlated with enhanced inhibition (Song and Messing, 2005; Mou et al., 2011). In consequence, neonatal ionizing radiation exposure might induce a rise in GABAergic neurotransmission, probably through PKCß1 activation, that could trigger a compensatory mechanism aimed to restore the inhibitory tone. Present results support this hypothesis, given that simultaneous increases in PKCß1, GAD and $GABA_AR$ levels were found. Therefore, it could be postulated that an adaptive response might overcome a sudden alteration of the GABAergic transmission, leading to the restoration of homeostatic inhibition.

In consequence, the formative pathways during development that contribute to normal anxiety-related behavior later in life might be blocked, attenuated or disorganized in different disorders or under the influence of physical agents such as ionizing radiation. Finally, the increase in $GABA_AR$ density and affinity as well as the enhancement in GAD induced in the HC of irradiated animals, triggered by the increase in PKCß1, might determine the lower anxiety levels.

5. Conclusions

In conclusion, these data suggest that PKCß1 might constitute a primary target for neonatal radiation damage on the hippocampus. Therefore, it could be hypothesized that an initial decrease in the levels of this protein can trigger a subsequent compensatory increase that, in turn, could be responsible for the plethora of biochemical changes that might underlie the previously observed behavioral alterations.

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References

Abramian AM, Comenencia-Ortiz E, Vithlani M, Verena Tretter E, Sieghart W, Davies PA, et al. Protein kinase C phosphorylation regulates membrane insertion of $GABA_A$ receptor subtypes that mediate tonic inhibition. J Biol Chem 2010;285(53):41795–805.
Adiga IK, Nair RR. Multiple signaling pathways coordinately mediate reactive oxygen species dependent cardiomyocyte hypertrophy. Cell Biochem Funct 2008;26(3):346–51.
Almeida M, Han L, Ambrogini E, Bartell SM, Manolagas SC. Oxidative stress stimulates apoptosis and activates NF-κB in osteoblastic cells via a PKCß/$p66^{shc}$ signaling cascade: counter regulation by estrogens or androgens. Mol Endocrinol 2010;24:2030–7.
Alzamora R, Harvey BJ. Direct binding and activation of protein kinase C isoforms by steroid hormones. Steroids 2008;73:885–8.
Bannerman DM, Rawlins JN, McHugh SB, Deacon RM, Yee BK, Bast T, et al. Regional dissociations within the hippocampus–memory and anxiety. Neurosci Biobehav Rev 2004;28(3):73–83.
Barros D, Amaral OB, Izquierdo I, Geracitano L, do Carmo Bassols Raseira M, Henriques AT, et al. Behavioral and genoprotective effects of Vaccinium berries intake in mice. Pharmacol Biochem Behav 2006;84(2):229–34.
Benes FM, Lim B, Matzilevich D, Walsh JP, Subburaju S, Minns M. Regulation of the GABA cell phenotype in hippocampus of schizophrenics and bipolars. Proc Natl Acad Sci U S A 2007;104(24):10164–9.
Biggio F, Gorini G, Caria S, Murru L, Sanna E, Follesa P. Flumazenil selectively prevents the increase in a4-subunit gene expression and an associated change in GABAA receptor function induced by ethanol withdrawal. J Neurochem 2007;102:657–66.
Brandon NJ, Uren JM, Kittler JT, Wang H, Olsen R, Parker PJ, et al. Subunit-specific association of protein kinase C and the receptor for activated C kinase with GABA type A receptors. J Neurosci 1999;119(21):9228–34.
Brandon NJ, Delmas P, Kittler JT, McDonald BJ, Sieghart W, Brown DA, et al. GABAA receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. J Biol Chem 2000;275(49):38856–62.
Brandon NJ, Jovanovic JN, Smart TG, Moss SJ. Receptor for activated C kinase-1 facilitates protein kinase C-dependent phosphorylation and functional modulation of GABA(A) receptors with the activation of G-protein-coupled receptors. J Neurosci 2002;22(15):6353–61.
Caceres LG, Aon Bertolino L, Saraceno GE, Zorrilla Zubilete MA, Urán SL, Capani F, et al. Hippocampal-related memory deficits and histological damage induced by neonatal ionizing radiation exposure. Role of oxidative status. Brain Res 2010;1312:67–78.
Caceres LG, Uran SL, Zorrilla Zubilete MA, Romero JI, Capani F, Guelman LR. An early treatment with 17-β-estradiol is neuroprotective against the long-term effects of neonatal ionizing radiation exposure. J Neurochem 2011;118(4):626–35.
Cammarota M, Paratcha G, Levi de Stein M, Bernabeu R, Izquierdo I, Medina JH. B-50/GAP-43 phosphorylation and PKC activity are increased in rat hippocampal synaptosomal membranes after an inhibitory avoidance training. Neurochem Res 1997;22(4):499–505.
Cid MP, Salvatierra NA, Arce A. Phosphatidylinositol 4,5-bisphosphate induced flunitrazepam sensitive-GABAA receptor increase in synaptosomes from chick forebrain. Neurochem Res 2007;32:1011–5.
Cid MP, Arce A, Salvatierra NA. Acute stress or systemic insulin injection increases flunitrazepam sensitive-GABAA receptor density in synaptosomes of chick forebrain: modulation by systemic epinephrine. Stress 2008;11(2):101–7.
Cid MP, Vilcaes AA, Rupil LL, Salvatierra NA, Roth GA. Participation of the GABAergic system on the glutamate release of frontal cortex synaptosomes from Wistar rats with experimental autoimmune encephalomyelitis. Neuroscience 2011;189:337–44.
Clarkson AN, Huang BS, MacIsaac SE, Mody I, Carmichael ST. Reducing excessive GABAergic tonic inhibition promotes post-stroke functional recovery. Nature 2010;468(7321):305–9.
Dai X, Cao X, Kreulen DL. Superoxide anion is elevated in sympathetic neurons in DOCA-salt hypertension via activation of NADPH oxidase. Am J Physiol Heart Circ Physiol 2006;290:H1019–26.
Depino AM, Tsetsenis T, Gross C. GABA homeostasis contributes to the developmental programming of anxiety-related behavior. Brain Res. 2008;1210:189–99.
Driver AS, Kodavanti PR, Mundy WR. Age-related changes in reactive oxygen species production in rat brain homogenates. Neurotoxicol Teratol 2000;22(2):175–81.
Femenía T, Gómez-Galán M, Lindskog M, Magara S. Dysfunctional hippocampal activity affects emotion and cognition in mood disorders. Brain Res 2012;1476:58–70.
Fushiki S. Radiation hazards in children - lessons from Chernobyl, Three Mile Island and Fukushima. Brain Dev 2013;35(3):220–7.

Fuss J, Ben Abdallah NM, Hensley FW, Weber KJ, Hellweg R, Gass P. Deletion of running-induced hippocampal neurogenesis by irradiation prevents development of an anxious phenotype in mice. PLoS One 2010;5(9):e12769.
Guelman LR, Zorrilla ZMA, Rios H, Ziehe LM. WR-2721 (amifostine, ethyol®) prevents motor and morphological changes induced by neonatal X-irradiation. Neurochem Int 2003;42:385–91.
Hines RM, Davies PA, Moss SJ, Maguire J. Functional regulation of $GABA_A$ receptors in nervous system pathologies. Curr Opin Neurobiol 2012;22(3):552–8.
Huleihel R, Yanai J. Disruption of the development of cholinergic-induced translocation/activation of PKC isoforms after prenatal heroin exposure. Brain Res Bull 2006;69(2):174–81.
Inoguchi T, Li P, Umeda F, Yu HY, Kakimoto M, Imamura M, et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C–dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes 2000;49:1939–45.
Kash SF, Tecott LH, Hodge C, Baekkeskov S. Increased anxiety and altered responses to anxiolytics in mice deficient in the 65-kDa isoform of glutamic acid decarboxylase. Proc Natl Acad Sci U S A 1999;96:1698–703.
Kimler BF. Prenatal irradiation: a major concern for the developing brain. Int J Radiat Biol 1998;73:423–34.
Lewis DA, Hashimoto T, Volk DW. Cortical inhibitory neurons and schizophrenia. Nat Rev Neurosci 2005;6(4):312–24.
Maher P. How protein kinase C activation protects nerve cells from oxidative stress-induced cell death. J Neurosci 2001;21(9):2929–38.
Majewski H, Iannazzo L. Protein kinase C: a physiological mediator of enhanced transmitter output. Prog Neurobiol 1998;155(5):463–75.
Martín-García E, Darbra S, Pallarés M. Neonatal finasteride induces anxiogenic-like profile and deteriorates passive avoidance in adulthood after Intrahippocampal neurosteroid administration. Neuroscience 2008;154:1497–505.
Mou L, Heldt SA, Ressler KJ. Rapid brain-derived neurotrophic factor-dependent sequestration of amygdala and hippocampal $GABA_A$ receptors via different tyrosine receptor kinase B-mediated phosphorylation pathway. Neuroscience 2011;176:72–85.
Palgi Y, Ben-Ezra M, Aviel O, Dubiner Y, Baruch E, Soffer Y, et al. Mental health and disaster related attitudes among Japanese after the 2011 Fukushima nuclear disaster. J Psychiatr Res 2012;46(5):688–90.
Paratcha G, Furman M, Bevilaqua L, Cammarota M, Vianna M, de Stein ML, et al. Involvement of hippocampal PKCbetaI isoform in the early phase of memory formation of an inhibitory avoidance learning. Brain Res 2000;855(2):199–205.
Phelps EA. Human emotion and memory: interactions of the amygdala and hippocampal complex. Curr Opin Neurobiol 2004;14(2):198–202.
Song M, Messing RO. Protein kinase C regulation of GABAA receptors. Cell Mol Life Sci 2005;62:119–27.
Tien-Hsiung K, Yih-Jing L, Su-Jane W, Chen-Hua F, Lu-Tai T. Effect of honokiol on activity of GAD_{65} and GAD_{67} in the cortex and hippocampus of mice. Phytomedicine 2011;18(13):1126–9.
Tomimatsu N, Arakawa Y. Protein kinase C-mediated protection of motoneurons from excitotoxicity. Neurosci Lett 2008;439(2):143–6.
United Nations Scientific Commitee of the Effects of Atomic Radiation (UNSCEAR). Radiation effects on the developing human brain. Report to the general assembly, sources, effects and risks of ionizing radiation. USA: United Nations Publications; 1993. p. 805–42. [annex H].
Vianna MR, Alonso M, Viola H, Quevedo J, de Paris F, Furman M, et al. Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. Learn Mem 2000a;7(5):333–40.
Vianna MR, Barros DM, Silva T, Choi H, Madche C, Rodrigues C, et al. Pharmacological demonstration of the differential involvement of protein kinase C isoforms in short- and long-term memory formation and retrieval of one-trial avoidance in rats. Psychopharmacology (Berl) 2000b;150(1):77–84.
Wu J, Song TB, Li YJ, He KS, Ge L, Wang LR. Prenatal restraint stress impairs learning and memory and hippocampal PKCbeta1 expression and translocation in offspring rats. Brain Res 2007;1141:205–13.
Zhang F, Li C, Wang R, Han D, Zhang QG, Zhou C, et al. Activation of GABA receptors attenuates neuronal apoptosis through inhibiting the tyrosine phosphorylation of NR2A by Src after cerebral ischemia and reperfusion. Neuroscience 2007;150(4):938–49.