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Short Communication

Growth Hormone Increases Neural Cell Adhesion Polysialylation State in the Dentate Gyrus of γ-Irradiated Rats

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INTRODUCTION

Prenatal irradiation has severe consequences in the central nervous system (CNS), ranging from microcephaly and subcortical heteropia to cognitive dysfunction in rodents (Schull and Otake, 1991; Schull et al., 1990). Radiation exposure induces cerebral cortex malformation, mental retardation, and attention deficit-hyperactivity disorders in humans (Mizumatsu et al., 2003; Monje, 2008; Schull and Otake, 1991; Schull et al., 1990); in adult γ-irradiated rats, it causes memory impairment (Sienkiewicz et al., 1994).

Neural cell adhesion molecule (NCAM) is the major carrier of 2,8-linked sialic acid residues (PSA), which are only present in regions of synaptic plasticity and permanent neurogenesis in the adult rat brain. NCAM is involved in synaptogenesis, neuroprotection, axonal sprouting and, memory formation (Cremer et al., 2000; Merino et al., 2000). Polysialic acid (PSA) is a posttranslational modification of NCAM that promotes plasticity and allows structural remodeling in the brain (Kiss and Rougon, 1997).

Different products have been used against the detrimental effects of gamma radiation in rodents, including growth hormone (GH) (Gómez de Segura et al., 1998; Madrid et al., 2002). Several studies have identified GH-receptors in the hippocampus and the prefrontal cortex (Lobie et al., 1993; Zhai et al., 1994). The pleiotropic actions of GH in the CNS range from neuroprotection, neurogenesis (Scheepens et al., 2001), axonal elongation, and dendritic arborization, to neuronal migration, and it can reduce cognitive impairment (Aberg et al., 2000; Muresanu et al., 2006; Sienkiewicz et al., 2000).

Because these brain processes are regulated by PSA-NCAM and hormones, including GH, we wanted to ascertain whether PSA-NCAM might be a target for GH effects in the dentate gyrus of γ-irradiated rats. Few studies have investigated the relationship between cell adhesion molecules and the effect of GH in the hippocampus of γ-irradiated rats (Hienz et al., 2008). The possibility that GH induces remodelatory effects and/or lessens cell death in the hippocampus of γ-irradiated rats through a regulation of PSA-NCAM protein levels has not been investigated. To answer these questions, we evaluated PSA-NCAM levels by immunofluorescence and CXCR4 by immunoblot in the hippocampus of γ-irradiated either treated or not treated with GH. We also analyzed whether GH exerts antioxidant effects on SOD-2-*Mitochondrial Superoxide Dismutase 2*-dependent levels as well as whether GH might regulate CXCR4 protein levels in the hippocampus of γ-irradiated rats.

MATERIALS AND METHODS

Animals

Experiments were performed with young adult adult male *Wistar* rats (p80, $n = 7$ rats per group, Experimental Surgery Dept. "*La Paz*" *Universitary Hospital, Madrid*) weighing 250–280 g. Rats were housed in groups of two animals per cage and maintained at 21°C with a 12-h light–dark cycle and free access to food and water. Radiated + GH rats received radia-

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tion and GH daily via i.p (i.p., 1 mg/kg body weight) injection (*Rad + GH*). Radiated rats received 6 Gy irradiation but no GH (*Rad*). Untreated control animals received no irradiation and no GH (*Cont*).

METHODS

Protocol: GH injection in γ-irradiated rats

Rats were anesthetized with isofluorane 2% and irradiated with 6 Gy in the whole body at 2 Gy/min in an *SLI* (Phillips accelerator). On day 1, the rats received light ether anesthesia and were placed in a supine position. A 4.9 × 5.1 cm field was used with a source to skin distance of 100 cm for a whole body irradiation. Radiation was delivered by photons of 6 meV from a linear accelerator (Elecka, SLI). The average calculated rat dose at the center of the field was 450 cGy/min. Previous studies have shown these parameters to be reliable and reproducible to study the effects of radiation enteritis without mortality until the fourth day after radiation, at which time the progressive effects of mucosal atrophy and ulceration are significant (Alexandrides et al., 1998).

GH treatment was administered for 4 consecutive days ending on the day of γ-radiation exposure (GH Rad group). GH (*Phytzer*) was diluted in PBS, and the hormone was injected at 1 mg/kg for 4 consecutive days (3 days before irradiation as well as on the same day as the gamma irradiation and then for 4 more consecutive days). Irradiated controls did not receive GH treatment and another control group without any treatment—GH or radiation—was also included in the experimental design. Immediately after the last radiation session, rats were sacrificed by decapitation, and the brains were removed. PSA-NCAM levels were analyzed by immunofluorescence in the hippocampus, and SOD-2 and CXCR4 were tested by immunoblot.

SOD-2 analyses by Western blot

SOD-2 and CXCR4 levels were tested in synaptosomes from hippocampus by immunoblot. Briefly, these synaptosomes were boiled at 90°C in 30 mM Tris–HCl buffer (pH 7.4) containing 0.05% SDS (Lauryl dodecyl sodium buffer) and β-mercaptoethanol. Equal amounts of proteins were loaded (30 μg) and separated on 10% SDS–PAGE polyacrylamide gels (w/v) (Merino et al., 2000). Gels were transferred to PVDF membranes at 1 mA/cm^2 (1 Å, 200 V during 1 h in TE 22 Transfer System, Amersham, Spain). Prestained kaleidoscope standard markers (*Bio-Rad 161-034*) detected an 18 kDa band for SOD-2 (AbCAM), a 45 kDa band for CXCR4 (Santa Cruz Biotechnology, Spain) as well as a 42 kDa band for beta actine (Sigma, Spain) following stripping protocols. Bands were normalized with beta actine. After three washes in TBS-Tween, blots were incubated with anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Scbt 1:20,000) for 2 h at room temperature (RT). Blots were washed with TBS-Tween for 5 min and the signal enhanced by incubation with Streptavidin-peroxidase conjugated solution diluted at 1:10,000 in PBS 1× for 30 min at RT (Merino et al., 2008). Finally, blots were washed three times with TBS-Tween 20 1×, and bands were developed with the ECL$^+$ chemoluminiscence system (Amersham Pharmacy, # RPN2132) and analyzed using Bio-Rad software.

Quantification of PSA-NCAM levels by immunoflorescence in the rat hippocampus

The brain hemisphere was postfixed in 4% paraformaldehyde (4%) at RT for 4 h. Immediately after postfixation brains were cryoprotected by immersion in a 30% sucrose solution for 24 h at 4°C. Brains were cut in the hippocampus at 12 μm with the cryostat and samples permeabilized by incubation in PBS 1× plus 1% Triton X-100 for 15 min (Watson and Paxinus, 1997). After blocking unspecific binding by incubation with 5% normal donkey serum (# 017-000-001, Jackson Immunoresearch), sections were overnight incubated with primary antimouse monoclonal Goat Ig M antibody (1:300) raised against polysialic acid-neural cell adhesion molecule (PSA-NCAM; clone 2-2B, Abcys 0019, France), which recognizes PSA residues longer than 12 neuraminic acid residues from NCAM (Cremer et al., 2000; Merino et al., 2000, 2008). We incubated hippocampal slices with this monoclonal antibody for 18 h at 1:300 in PBS 1× buffer containing 0.03% BSA at RT (Sandi et al., 2003). For PSA-NCAM immunodetection, we incubated them with a conjugated Goat antimouse-FITC Ig M secondary antibody (Calbiochem, #401224) diluted at 1:300 in PBS1× buffer. PSA-NCAM specificity was confirmed by endoneuraminidase (Endo N, Abcys, France) treatment, which removes *PSA* from *NCAM* (Merino et al., 2009).

Quantification of neuronal loss in the dentate gyrus and hippocampal CA3 area

We also compared the number of neurons in the dentate gyrus and CA3 of rats receiving GH to the number in untreated control irradiated rats using the Cavalieri principle (Paxinus and Watson, 1997; Segovia et al., 2009). Hematoxylin-eosin staining was evaluated in 30-μm-thick paraffin-embedded samples in the hippocampus.

Statistical analysis

Data obtained were expressed as mean ± S.D following a two-way analysis of variance (ANOVA) and post hoc Fisher's test and *t*-Student post hoc analyses. Significance was set at $P < 0.05$.

RESULTS

The observed data showed increased PSA-NCAM levels in GH-irradiated rats compared to γ-irradiated

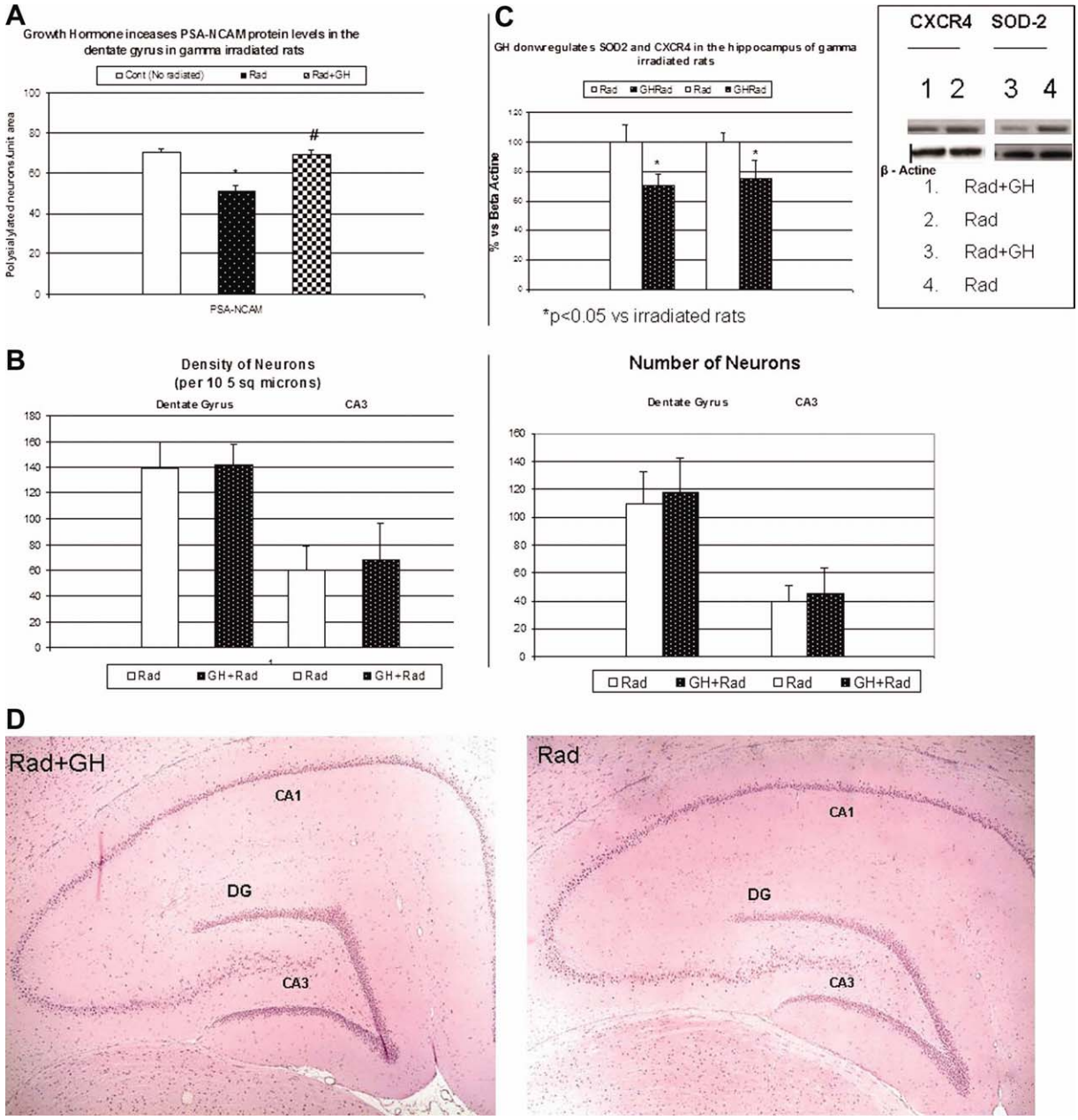

Fig. 1. **A:** Increased NCAM polysialylated state in the dentate gyrus of GH gamma irradiated rats (n = 7–8, Rad + GH) in comparison with gamma-radiated rats ($P < 0.05$ vs. irradiated animals, Rad). Cont = undisturbed rats (non irradiated control animals, immunofluorescence images in Figure 2). Rad receiving gamma radiation. GH + Rad = Irradiated GH-treated rats. **B:** Morphometry in the dentate gyrus and CA3 area from hippocampus of irradiated and hGH irradiated rats. The average neurons density in the CA3 area shows a slight trend to increase in the granular cell layer as a consequence of GH treatment (Fig. 1, $P < 0.11$; n.s). **C:** Mean values for CXCR4 and SOD-2 in Western blots from irradiated GH rats (Rad + GH) and control irradiated animals (Rad) (n = 7 per group). **D:** Hematoxilin-eosin staining in the hippocampus (DG, dentate gyrus, CA1 and CA3). Rad = irradiated rats and GH gamma radiated animals (Rad + GH). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

animals. In addition, ANOVA showed that radiation reduces the NCAM polysialylaton state in comparison to control nonradiated animals ($F_{2,23}$ = 11.62; $P < 0.05$). The Student's t test indicated a slight trend to neuronal protection against radiation in the CA3 area in GH-irradiated rats when compared with radiated controls ($P < 0.1$; n.s; Figs. 1A, 1B, and 2). Additionally, the t test confirmed the reduced CXCR4 and SOD-2 levels in the Western blots from the hippocampus of γ-irradiated GH-treated rats in comparison with irradiated animals ($P < 0.05$, Fig. 1C).

DISCUSSION

The neuronal loss observed in the hippocampus of γ-irradiated controls in comparison with untreated

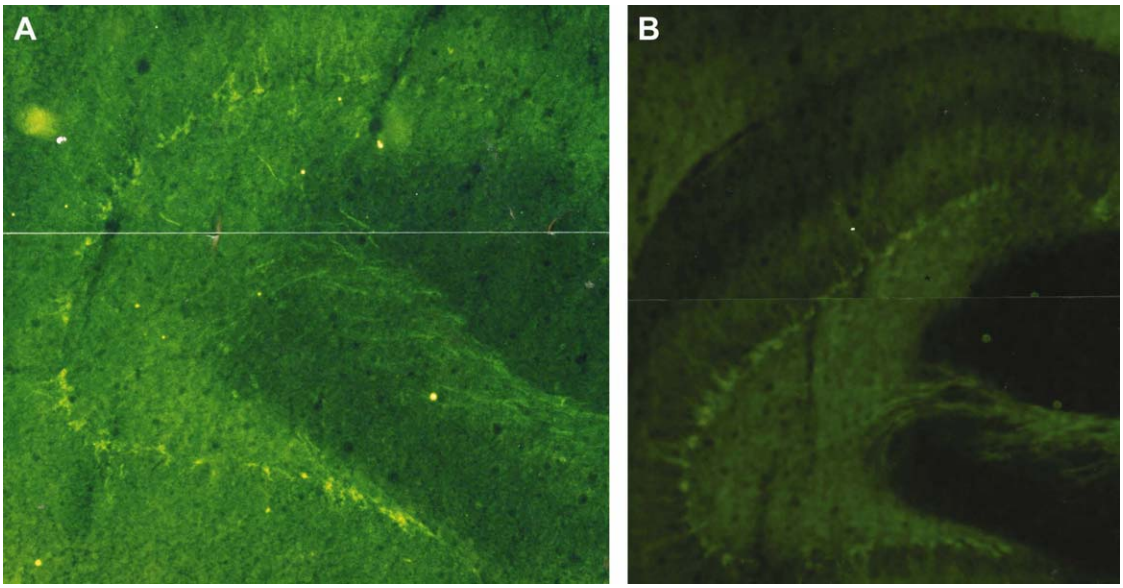

Fig. 2. **A:** PSA-NCAM in the dentate gyrus of gamma-irradiated rats. **B:** PSA-NCAM in the dentate gyrus of gamma-irradiated rats. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

control rats (no GH, no gamma irradiation) has also been reported elsewhere and was described as ventricular dilation (Sienkiewicz et al., 2000). Because PSA-NCAM is involved in dendritic arborization, axonal elongation, neurogenesis, and memory formation (Cremer et al., 2000; Merino et al., 2000, 2003) and is a member of the same immunoglobulin superfamily as L-1, which has been reported in the hippocampus of GH γ-irradiated rats (Sun et al., 2002), we suggest that PSA-NCAM upregulation could have a remodelatory effect in this brain area in these animals (Sun et al., 2002). Thus, fetal mouse X-ray exposure is known to induce extensive neuronal loss in the hippocampus and also increase the incidence of neurological dysfunction in mice lacking the L1 cell-adhesion molecule that were trained in a spatial learning paradigm (Zhang et al., 2007). We believe that our GH treatment prevented detrimental effects from gamma radiation by increasing PSA-NCAM protein levels in the dentate gyrus (Fig. 1; Abbot et al., 2006). If we consider that radiation impairs neurogenesis and activates microglia in the hippocampus of γ-irradiated rats (Monje, 2007; Monje et al., 2007), the detected PSA-NCAM upregulation may preserve dendritic arborization in the dentate gyrus of GH-irradiated rats (see Figs. 1A, 2A, and 2B). Interestingly, Zhang et al. (2007) reported behavioral deficits in irradiated mice lacking the L1 cell adhesion that were trained in a hippocampus-dependent paradigm (Zhang et al., 2007). Because L1 is another cell adhesion molecule that belongs to the superimmunoglobulin family, the possibility that PSA-NCAM induces neuroplastic effects in the hippocampus of γ-irradiated rats cannot be excluded (Isgaard et al., 2007). GH has several known protective effects in brain pathological conditions ranging from excytotoxicity to inflammation and hypoxia (Mitsunaka et al., 2001; Muresanu and Sharma, 2007; Scheepens et al., 2001). Interestingly, Duveau et al. (2007) reported that the presence of PSA seemed to be functionally linked to neuroprotection in the hippocampus after kainate-induced cell death that was abolished in the absence of NCAM polysialylation (Duveau et al., 2007). In fact, neuroprotective effects from NCAM have been associated to several pathological conditions in the hippocampus (Mikkonen et al., 2003). Therefore, increased PSA-NCAM levels in the hippocampus may be linked to remodelatory effects in the dentate gyrus (Merino et al., 2000) of γ-irradiated rats receiving GH treatment before radiation.

On the other hand, the SOD-2 reduction reported in GH-treated rats may itself exert antioxidant effects in the hippocampus of γ-irradiated rats. From a chemotactic view point, chemokines, including CXCR4 alpha chemokine receptor, are thought to be essential modulators of tissue inflammation, injury, and repair through the recruitment of leukocytes into *stressed* host tissues (Asensio and Cambell, 2000; Merino et al., 2009). Although our data do not allow us to analyze whether leukocyte recruitment may be modulated by CXCR4 chemokine levels in the hippocampus of γ-irradiated animals, Moriconi et al. (2008) reported that radiation increased CXCR4 at the transcriptional level in peripherical organs of irradiated rodents (Moriconi et al., 2008). Interestingly, Merino et al. (2008) reported that PSA-NCAM is a target of chemokines in cortical neurons in vitro (Merino et al.,

2008, 2009). In conclusion, our data suggest that augmented PSA-NCAM levels may prevent dendritic retraction in the granular cell layer of γ-irradiated rats (Zhang et al., 2007) and that CXCR4 chemokine receptor may be a target of GH protective effects in the hippocampus of γ-radiated rodents. Further studies will evaluate whether GH induces neurogenesis and amilorates leukocyte recruitment through PSA-NCAM/CXCR4 modulation in the injured hippocampus of γ-irradiated rats.

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