

Research article

Expression of RAD9B in the mesostriatal system of rats and humans: Overexpression in a 6-OHDA rat model of Parkinson's disease



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ABSTRACT

Background: Parkinson's disease (PD) is a neurodegenerative disorder that affects primarily the dopaminergic (DAergic) neurons of the mesostriatal system, among other nuclei of the brain. Although it is considered an idiopathic disease, oxidative stress is believed to be involved in DAergic neuron death and therefore plays an important role in the onset and development of the disease. RAD9B is a paralog of the RAD9 checkpoint, sharing some similar functions related to DNA damage resistance and apoptosis, as well as the ability to form 9–1–1 heterotrimer with RAD1 and HUS1.

Methods: In addition to immunohistochemistry, immunofluorescence and Western-blot analysis, we implemented Quantitative RT-PCR and in situ hybridization techniques.

Results: We demonstrated RAD9B expression in rat and human mesencephalic DAergic cells using specific markers. Additionally, we observed significant overexpression of RAD9B mRNA ($p < 0.01$) and protein ($p < 0.01$) in the midbrain 48 h after inducing damage with 150 μ g of 6-hydroxydopamine (6-OHDA) injected in a rat model of PD. Regarding protein expression, the increased levels were observed in neurons of the mesostriatal system and returned to normal 5 days post-injury.

Conclusions: This response to a neurotoxin, known to produce oxidative stress specifically on DAergic neurons indicates the potential importance of RAD9B in this highly vulnerable population to cell death. In this model, RAD9B function appears to provide neuroprotection, as the induced lesion resulted in only mild degeneration. This observation highlights the potential of RAD9B checkpoint protein as a valuable target for future therapeutic interventions aimed at promoting neuroprotection.

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1. Introduction

RAD9B is a checkpoint paralog of the RAD9 gene, sharing a similar amino acid profile. It was originally described in humans and mice by Default et al. in 2003. Both RAD9 (also referred to as RAD9A) and RAD9B are distinct protein isoforms encoded by the RAD9 gene in various species (Hopkins et al., 2003; Hwang et al., 2022). RAD9 functions are mainly related to its role in DNA repair, apoptosis and

conferring resistance to DNA damage (Lieberman, 2006; Lieberman et al., 2011; Ferrari et al., 2020). The involvement of RAD9 in the normal functioning of embryonic development has also been previously demonstrated in mouse models (Hopkins et al., 2004; Huang et al., 2019). RAD9 forms a heterotrimer with RAD1 and HUS1 proteins, known as 9–1–1, which is an analogue of the proliferating cell nuclear antigen (PCNA) homotrimeric DNA clamp (Doré et al., 2009; Mascarenhas Dos Santos et al., 2022). Both PCNA and 9–1–1 function as scaffolds for the recruitment of proteins required for DNA repair and cell cycle control (Querol-Audí et al., 2012). RAD9B has been suggested to participate in RAD9-like functions due to its ability to form heterotrimers by associating with RAD1 and HUS1, or in addition to its paralog HUS1B, forming an alternative 9–1–1 complex

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(Dufault et al., 2003; Pereira et al., 2022). RAD9B has been poorly studied, but several specific functions have been attributed to it. It has been proposed to be involved in genome maintenance during meiosis in mammals, due to its ability to form 9–1–1 complexes (Lyndaker et al., 2013). Like RAD9, RAD9B has also been observed to be critical for embryonic development and for conferring resistance to specific types of DNA damage (Leloup et al., 2010). In its role as a gene checkpoint, it is known to be required for the nuclear localisation of claspin protein; an adaptor required for DNA replication (Sierant et al., 2010) and involved in stress response and nucleolar function (Pérez-Castro and Freire, 2012).

Parkinson's disease (PD) is the most common movement disorder and the second most prevalent neurodegenerative pathology worldwide, affecting 1–2 out of every 1000 inhabitants and reaching 1% of the population over 60 years of age (Samii et al., 2004; Tysnes and Storstein, 2017; Tolosa et al., 2021). Studies suggest that the onset of the degenerative process in PD occurs as a result of the accumulation of α -synuclein and the consequent development of Lewy bodies in the olfactory bulb and the dorsal nucleus of the vagus, before spreading to the rest of the affected regions (Braak et al., 2004; Klingelhöfer and Reichmann, 2015; Bove and Travaglini, 2019). The most obvious clinical manifestations used to diagnose this pathology are resting tremor, bradykinesia and rigidity (Berardelli et al., 2001; Sveinbjornsdottir, 2016; Reich and Savitt, 2019), resulting from impaired basal ganglia function due to the mesencephalic DAergic neurons loss in the substantia nigra pars compacta (SNc) (Trist et al., 2019; Pajares et al., 2020; Vázquez-Vélez and Zoghbi, 2021). The vulnerability of DAergic neurons differs depending on their localisation; those situated in the substantia nigra (SN) (A9 cell group) exhibit a higher susceptibility to degeneration compared to those in the ventral tegmental area (VTA) (A10 cell group) (Dahlström and Fuxe, 1964). The degree of degeneration of these cells in PD also varies within the SN, with DAergic neurons in the ventrolateral and caudal regions (SNcv) being more vulnerable than those in the rostromedial and dorsal regions (SNrm) (Damier et al., 1999; Afonso-Oramas et al., 2010). A wide variety of animal models have traditionally been used to study different PD-associated abnormalities, ranging from non-human primates to rodents and even *Drosophila* (Shyh et al., 2020). While the use of genetic models involving the manipulation of PD-related genes such as α -synuclein, leucine-rich repeat kinase 2 (LRRK2), parkin (PRKN), PTEN-induced kinase 1 (PINK1) or protein deglycase (DJ-1) (Dawson et al., 2010; Zhang et al., 2022) has significantly advanced the study of the disease, neurotoxin-induced models have been fundamental for a better understanding of PD-associated motor disorders. Compounds commonly used to produce PD-like degeneration in animal models are 6-OHDA, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat and rotenone. 6-OHDA has a high affinity for the dopamine transporter (DAT) due to its high similarity to dopamine and is the most commonly used neurotoxin in rat models of PD (Bové and Perier, 2012; Blesa et al., 2012). 6-OHDA molecule has to be injected directly into the brain due to the difficulty of crossing the blood-brain barrier, and after entering the DAergic cell using DAT, drives cell death through the production of reactive oxygen species (ROS) (Cohen, 1984; Shen et al., 2017). MPTP is traditionally used in mouse and non-human primate models, and unlike 6-OHDA, has the ability to rapidly cross the blood-brain barrier after peripheral injection (Dionísio et al., 2021) and also produces ROS, leading to mesencephalic DAergic cells degeneration (Martí et al., 2017; Schildknecht et al., 2017). The use of pesticides, such as rotenone, which inhibits mitochondrial complex I, and paraquat, which causes oxidative stress, also results in the loss of DAergic neurons in the SN and PD-related behavioural changes (Tanner et al., 2011; Thirugnanam and Santhakumar, 2022). Therefore, these models support the hypothesis that ROS production and thus oxidative

stress play a very important role in the development of PD (Hemmati-Dinarvand et al., 2019; Niranjana, 2014).

The aim of this work has been divided into two parts: 1) To investigate the expression of RAD9B in the mesostriatal system of rats and humans. 2) To study RAD9B expression changes in mesencephalic DAergic cells using a rat model of PD inducing oxidative damage by injection of 6-OHDA.

2. Material and methods

2.1. Morphological study

The morphological study was conducted in healthy rats and humans and in a 6-OHDA-treated rat model of PD. Human brains were obtained from the Department of Basic Medical Sciences of the University of La Laguna and were authorised to be donated for scientific and educational purposes. Human brains were obtained according to national Spanish guidelines and under the supervision of the Ethics Committee of the University of La Laguna, in accordance with the Declaration of Helsinki, 1975. Experimental protocols in rats were approved by the Ethics Committees of the University of La Laguna (#CEIBA20130083) under the European Directive of 22 September 2010 (2010/63/EU) and the ARRIVE guidelines. Human brains were obtained from 4 donors (2 men and 2 women; average age 63.2 ± 4.4 years) who died without a history of drug abuse or neurological or psychiatric disease. Brains were removed after a post-mortem period of 16.3 ± 5.4 h. Blocks containing the entire DAergic formation of the midbrain (from the mammillary bodies to the pons) were selected. The pieces were briefly washed in PBS and immersed in 4% paraformaldehyde pH 7.4 in PBS for 72 h at 4 °C. They were then cryoprotected in a graded series of sucrose-PBS solutions and stored at -80 °C until processing. Thirty male Sprague-Dawley rats (300–350 g) (control and 6-OHDA injected) were used in these experiments. Animals were deeply anaesthetised with an overdose of pentobarbital sodium and transcardially perfused with heparinised ice-cold 0.9% saline (150 ml) followed by 4% paraformaldehyde pH 7.4 in PBS (450 ml). Brains were removed and midbrain blocks were stored in the same fixative at 4 °C overnight, cryoprotected in a graded series of sucrose-PBS solutions, and stored at -80 °C until processing. Rat brain perfusion and cryoprotection solutions were treated with 0.1% diethyl pyrocarbonate (DEPC) and autoclaved to inactivate RNase.

2.2. Animal model of PD

A 6-OHDA-injected rat model of PD was used in this study. Ten male rats (300–350 g) per group were injected into the third ventricle (midline, 2 mm posterior to bregma and 8 mm below dura mater, according to Paxinos and Watson, 1998) with vehicle (0.9% saline with 0.3 μ g/ μ l ascorbic acid; control group, $n = 10$) and with a single 150 μ g dose of 6-OHDA (6-hydroxydopamine hydrochloride, Sigma; in 7.5 μ l of vehicle per injection; 6-OHDA group, $n = 20$; for protocol details see Rodríguez et al., 2001 and Afonso-Oramas et al., 2010). Rats were sacrificed 48 h ($n = 10$) and 5 days ($n = 10$) after injury.

2.3. Tissue processing

Coronal sections (25 μ m in rat, 50 μ m in human) were obtained using a freezing microtome, collected in parallel series and processed for RAD9B and tyrosine hydroxylase (TH) immunostaining.

For single immunohistochemical (IHC) labelling, floating sections were immersed for 30 min in 3% H_2O_2 to inactivate endogenous peroxidase, and incubated for 60 min at room temperature (RT) in 4% normal goat serum (NGS; Jackson ImmunoResearch, West Grove, PA) in PBS, containing 0.05% Triton X-100 (TX-100; Sigma). The

sections were then incubated overnight in PBS containing 2% NGS and the primary antibody: rabbit anti-RAD9B polyclonal antibody (1:2000) produced, tested and published by Dr Raimundo Freire's group (Pérez-Castro and Freire, 2012). After several rinses, the sections were incubated for 2 h in either biotinylated goat anti-rabbit antiserum (1:200; Jackson ImmunoResearch) or 1:200 NGS in PBS. Immunoreactions were visualised after incubation in ExtrA-vidin-peroxidase (1:5000; Sigma) in PBS for 1 h at RT, followed by 10 min in 0.005% 3'-3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.001% H₂O₂ in 0.05 N cacodylate buffer pH 7.6. Rat midbrain sections were also processed for double immunolabelling using rabbit anti-RAD9B polyclonal antibody (1:2000) and mouse anti-TH monoclonal antibody (Sigma, 1:12,000). After preincubation in 4% NGS, 4% NDS and 0.05% TX-100 in PBS, they were incubated overnight with both antibodies. Immunofluorescence labelling was revealed after incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:200; Molecular Probes, Eugene, OR) and biotinylated conjugated goat anti-rabbit (1:200; Jackson ImmunoResearch) for 3 h, followed by Cy3-conjugated streptavidin (1:1000; Jackson ImmunoResearch) for 2 h at RT. After several rinses in PBS, midbrain coverslips were mounted with Vecta-shield Mounting Medium containing DAPI (Southernbiotech, Birmingham, AL).

2.4. Densitometric analysis

The signal intensity of the staining in rat mesencephalic sections was analysed. The study was performed on 5 rats per group, and six sections of 150 µm thickness, 150 µm apart, per rat. For the image acquisition, we used the optical dissection method and a computer-assisted image analysis system attached to an Olympus microscope (BX51) equipped with a 40 × , 0.75 N.A. objective, a motorised stage with serial optical Section 2 µm apart in the z-axis, and an Olympus camera (DP70). An electronic microcaptor (MT12) was connected to both the microscope and the parallel port of the computer. The labelling intensity of each cell was compared to the adjacent background in axodendritic-stained free areas, and a minimum of 60 cells per nucleus and rat were analysed. Microscopic images were digitised and the labelling intensity was quantified using densitometry software (Leica Microsystems, Wetzlar, Germany). The labelling intensity was defined as the index of light attenuation with respect to the neighbouring background and expressed as arbitrary units. Immunofluorescence was examined using a confocal laser scanning microscopy system with appropriate filters (RRID: SC_R_0168840; Olympus FV1000, Hamburg, Germany). The labelling intensity of each immunofluorescent marker was quantified using the ImageJ 1.53c programme. The study was performed on six 25-µm thick sections, spaced 150 µm apart from each other, per rat. Images were acquired under a 60 × oil objective (1024 × 1024 pixels resolution), considering 3 µm × 3 µm square areas and including soma and neurites. The fluorescent labelling intensity is expressed as a percentage of the fluorescence intensity observed in the control group.

2.5. Quantitative RT-PCR and Western-blot analysis

These studies were performed on control rats (n = 5) and rats injected with 150 µg 6-OHDA, 48 h (n = 5) and 5 days (n = 5) post-injection. Rats were anaesthetised and then sacrificed by decapitation, brains were promptly removed. Ventral mid-brains containing the SN (between 3.00 mm and 4.00 mm rostral to the interaural axis) were dissected in ice using a brain blocker. RNA and proteins were extracted using the acid phenol chloroform extraction method.

2.5.1. Quantitative RT-PCR

Reverse transcription was performed on 2 µg of total RNA after heat denaturation (65°C, 5 min) and annealing of oligo-dT primers

Table 1
Primers used in qPCR.

Genes	Forward (5'→3')	Reverse (5'→3')
RAD9B	TCCACATATGGCTACGTCCT	TGGCGACCATTTGACACTTC
RAD1	GCCTGAGGACACACTGGATT	CTTGTCAGGAGACACGGTGA
HUS1	AAGATCGTGGATCTGGCTTG	GTTCTCCTGCTCCAGCTCAC
Rps18	GTCATCCCCGAGAAGTTTCA	TTGGTGAGGTCAATGTCTGC

for quantitative RT-PCR (qPCR). AMV reverse transcriptase (Roche Diagnostics) was used for cDNA synthesis, under the conditions recommended by the manufacturer, in a final volume of 20 µl. The real-time assay was performed using a 96-well Opticon 2 cycler, the Opticon software version 3.1 (MJ Research, Waltham, MA), and the iQ™ SYBR® Green Supermix RT-PCR kit (Bio-Rad, Hercules, CA). Twenty-five microlitre reactions were pipetted into each well, containing 1 µl of DNA template, 0.2 µM of each primer, 12.5 µl of the SYBR® Green reagent and 11 µl of sterile Milli-Q water. Amplification conditions were as follows: (1) incubation step at 95 °C (3 min); (2) DNA amplification for 35 cycles of 94 °C (1 min), 55.5°C (45 s) and 72°C (45 s). Primer sequences for RAD9B, RAD1, HUS1 and the reference gene Rps18 are summarized in Table 1. Threshold cycles were calculated by plotting normalised fluorescence relative to cycle number, and the amplification efficiency of each primer pair was determined using standard cDNA dilutions. The normalised expression of each gene was calculated using the Q-Gene software (Muller et al., 2002). All assays were performed in triplicate and with five animals per group.

2.5.2. Western-blot

Protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as standard. Protein samples were diluted in Laemmli's loading buffer (62.5 mM Tris-HCl, 20% glycerol, 2% sodium dodecyl sulphate [SDS], 0.05% β-mercaptoethanol, and 0.05% bromophenol blue, pH 6.8), denatured (90 °C, 5 min), separated by electrophoresis in 10% SDS-polyacrylamide gel and transferred into nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Blots were blocked for 2 h at room temperature (RT) with 5% non-fat dry milk in TBST (250 mM NaCl, 50 mM Tris, pH 7.4%, and 0.05% Tween20) and incubated overnight at 4 °C in blocking solution with a rabbit anti-RAD9B polyclonal antibody (1:2000) (Pérez-Castro and Freire, 2012). After several rinses in TBST-5% milk, the membranes were incubated for 2 h at RT in horseradish peroxidase-conjugated anti-rabbit IgG (1:10,000; Jackson-ImmunoResearch). After probing for RAD9B protein, each nitrocellulose membrane was stripped (62.5 mM Tris, pH 6.8, 2% SDS, 100 mM β-mercaptoethanol; 1 h at RT) and subsequently re-probed for TH using a mouse monoclonal anti-TH antibody (Sigma; 1:10,000, 2 h at RT) or for r-Actin using a mouse anti-r-Actin antibody (Sigma; 1:10,000, 2 h at RT). Each immunoreactive band was compared with its respective loading control. Different protein loading levels, antibody dilutions, and exposure times were evaluated to set the working range of the antibodies. Immunoreactive bands were visualised using enhanced chemoluminescence (Immun-Star, Bio-Rad, CA) and a Chemi-Doc imaging system (Bio-Rad). Labelling densities were quantified using densitometry software (Image Lab 5.2; Bio-Rad). A rectangle of uniform size and shape was placed over each band, and the density values were calculated by subtracting the background at approximately 2 mm above each band. The identity of the protein extract was blinded for quantitative analysis of Western-blot images. Data are expressed as a percentage of average labelling intensity under basal conditions ± SEM.

2.6. In situ hybridization

RAD9B expression was studied by in situ hybridization (ISH) in rat midbrain tissue. The antisense and the control sense rat digoxigenin-labelled RAD9B riboprobes used in this study were produced by in vitro transcription of RAD9B cDNA in a dual-promoter expression vector. The RAD9B cDNA product was obtained using the following primers: sense, TGAGCGATGAACTGTGGCTA; and antisense, CGAGCTCTTGAGGCAGAAAT. The plasmid was linearised, affinity purified and transcribed with the appropriate RNA polymerases in order to synthesise either sense or antisense probes using commercial reagents (Roche). The 50 µl transcription mixture contained 1 µg template cDNA, 1 mM each of ATP, CTP and GTP, 0.7 mM UTP and 0.3 mM DIG-UTP, 10 mM DTT, 50 U RNase inhibitor and 1 U either T3 or T7 RNA polymerase. After 2 h at 37 °C, the template cDNA was digested with 2 U RNase-free DNase for 30 min at 37 °C. The riboprobes were precipitated by the addition of 100 µl 4 M ammonium acetate and 500 µl ethanol and centrifuged at 4 °C for 30 min. The quality of the synthesis was monitored by Northern-blot.

Midbrain floating sections were incubated twice with 0.1% active DEPC/PBS containing 2% (w/v) proteinase K for 15 min. After pre-equilibration with 5 × SSC (0.75 M NaCl and 0.075 M NaCitrate), the sections were pre-hybridized in hybridization solution (50% formamide, 5 × SSC, 40 µg/ml denatured salmon DNA) at 58 °C for 1 h. The probe was denatured (80 °C, 5 min) and added to the hybridization mix at 400 ng/ml. Sections were hybridized in this solution at 58 °C for 16 h. After post-hybridization washes, slides were equilibrated in TN buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and incubated for 2 h at RT with alkaline phosphatase-conjugated monoclonal anti-DIG antibody (1:5000 final dilution in TN with 0.5% blocking reagent, Roche Diagnostics). After washes in TN buffer, the slides were incubated for 8 h in chromogenic substrate (NBT and BCIP, Roche Diagnostics; 0.5%) in TNM buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂, pH 9.5). Staining was stopped in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Control experiments were performed by omitting the antisense riboprobe or using its control sense probe, resulting in no staining.

2.7. Statistical analysis

Mathematical analysis for the comparison of means was performed using Student's t-test and one-way ANOVA followed by the Tukey honest test for multiple post hoc comparisons. Analyses were performed using the Statistica software (Statsoft; Tulsa, USA). Data are expressed as mean ± SEM. A level of * $p < 0.05$ and ** $p < 0.01$ was considered critical for assigning statistical significance.

3. Results

3.1. RAD9B expression in rat and human mesostriatal system

We were able to demonstrate the expression of RAD9B protein in the rat and human midbrain by using the IHC technique with a specific antibody against RAD9B (Fig. 1 A-C). In both cases, we show that RAD9B labelling is present in the mesostriatal system. The presence of RAD9B labelling in the mesostriatal system of rats was corroborated by the RAD9B and TH double-labelling immunofluorescence (shown in the results section 3.2.2.). In humans, RAD9B expression in the mesostriatal system was comprehensively demonstrated by co-localising RAD9B labelling with neuromelanin-containing neurons (Fig. 1C); a dark pigment used as a histological marker of SN DAergic neurons (Nagatsu et al., 2023). Both rat and human tissue showing RAD9B expression were immunohistochemically labelled with and without the primary antibody (data not shown). Thus, we were able to demonstrate that

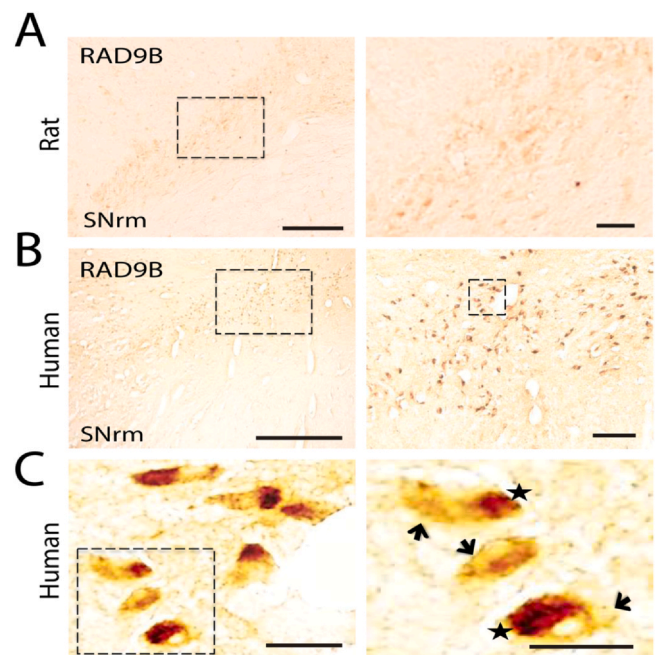


Fig. 1. A-C, Immunohistochemical staining of rat (A) and human (B-C) RAD9B in the ventral midbrain. The magnified view of the dashed box areas shows details of the neuronal labelling (right column). C, Magnified detail of RAD9B IHC labelling in human SN. Black stars indicate neuromelanin pigment deposits. Black arrows indicate the labelling detection with the RAD9B antibody. SNrm; substantia nigra rostro-medial. Scale bars: A 300 and 100 µm; B 500 and 100 µm; C 50 and 20 µm respectively.

RAD9B labelling was due to the interaction of the protein with the antibody and not an artefact of the technique. The expression levels observed in this section correspond to rats not subjected to a stress-inducing injury and to humans not diagnosed with PD. As shown in paragraph 2.2 of this results section, the RAD9B protein is over-expressed in rats following the application of a stressor and a more marked protein expression can be observed. Therefore, it should be noted that the RAD9B expression levels shown in Fig. 1 correspond to basal levels and although they are not as striking as those found in the 6-OHDA model, demonstrate the expression of RAD9B protein within a highly vulnerable region in PD.

3.2. Increased expression of RAD9B mRNA in the midbrain using ISH and qPCR techniques

ISH labelling of RAD9B increased in the midbrain of 6-OHDA-injected rats compared to controls (control vs 48 h, 100 ± 25 vs 194 ± 28 , $p < 0.01$) (Fig. 2 A-C). The increase in RAD9B mRNA expression occurs throughout the midbrain, showing in Fig. 2a rostral region. As RAD9B binds to different proteins, such as RAD1 or HUS1, to form complexes 9–1–1, required for its correct functioning, we performed experiments in the midbrain of rats to show the expression of these molecules at mRNA level by qPCR. An increase in the expression of RAD9B ($p < 0.05$) and RAD1 ($p < 0.05$), but not of HUS1, was observed in 6-OHDA-injected rats compared with the control (Fig. 2D, Table 2).

3.3. Increased expression of RAD9B protein in the mesostriatal system using IHC and Western-blot techniques

In line with the quantification of the RAD9B mRNA, RAD9B protein level was examined in the same 6-OHDA-injected rat model. RAD9B IHC was performed on midbrain sections from control and 150 µg 6-OHDA-injected rats at 48 h and 5 days post-injury (Fig. 3A-C). A significant increase in RAD9B labelling intensity was observed

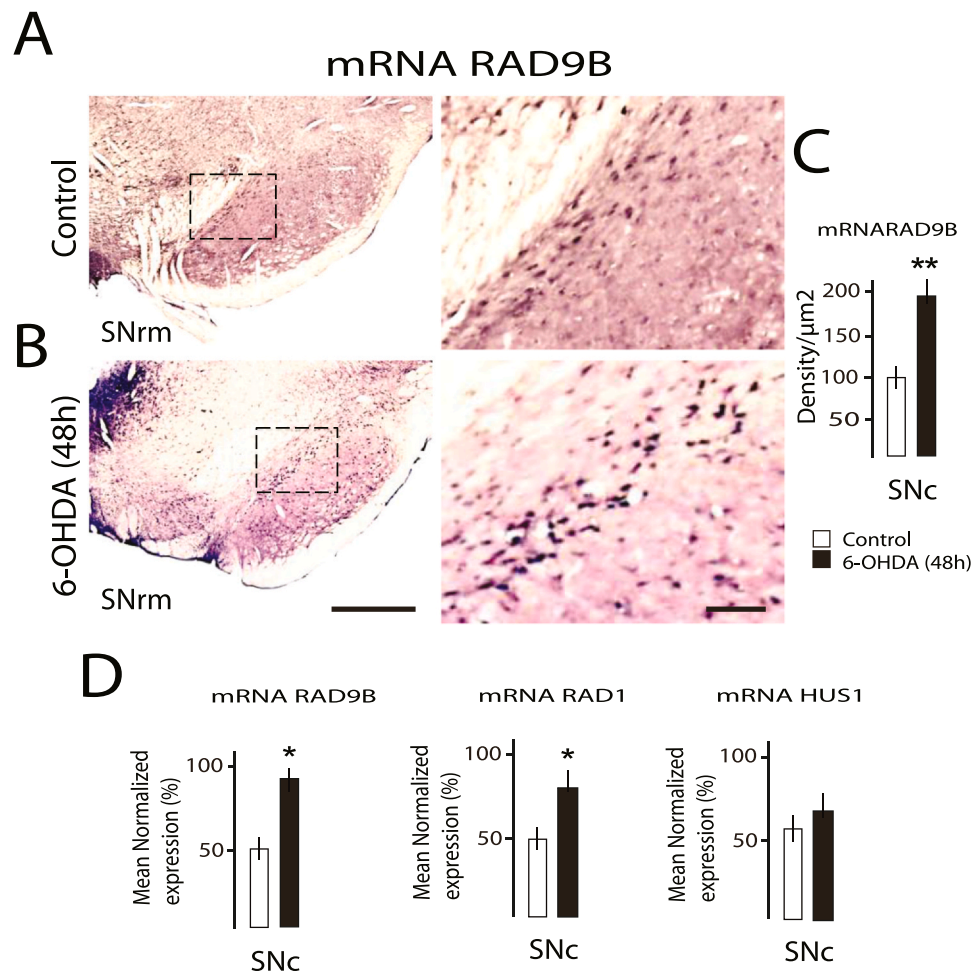


Fig. 2. A–B, Expression level of RAD9B mRNA by ISH using a specific probe in rat ventral midbrain. A, Expression of RAD9B in control rats (treated with vehicle). B, Expression of RAD9B in rats treated with 150 µg of 6-OHDA and sacrificed 48 h after injury. The dashed box represents the magnified area for densitometric analysis of RAD9B-positive neurons (right column). C, Histogram of the densitometric quantification of RAD9B ISH. Although the region of the mesencephalon corresponding to the area where the SNrm is located is shown, the study was performed along the entire SNc. D, Normalized mRNA levels of RAD9B, RAD1 and HUS1. * indicates $p < 0.05$, ** indicates $p < 0.01$. Bars, mean $\pm$ SEM. SNrm: substantia nigra rostro-medial; SNc: substantia nigra pars compacta. Scale bars: 500 and 100 µm.

Table 2
Analysis of Ct index in RAD9B, RAD1 and HUS1 qPCR. * $p < 0,05$ vs control.

	Control	150 µg 6-OHDA 48 h
Ct RAD9B/Ct Rps18	1254 $\pm$ 0024	0987 $\pm$ 0012*
Ct RAD1/Ct Rps18	1194 $\pm$ 0017	1074 $\pm$ 0024*
Ct HUS1/Ct Rps18	1127 $\pm$ 0035	1094 $\pm$ 0024

in the rat midbrain at 48 h after injury (control vs 48 h, 100 ± 20 vs 195 ± 22 , $p < 0.01$), with levels recovering 5 days after injection of 6-OHDA compared to controls (Fig. 3E). Protein overexpression was shown in a rostral region of the midbrain although it occurs throughout the whole midbrain. Double immunofluorescence labelling was performed using antibodies against RAD9B and TH in the different experimental conditions to confirm that the increase in RAD9B expression occurs in the DAergic neurons of the mesostriatal system (Fig. 4 A–B). Both SNrm and VTA regions are shown, revealing overexpression of RAD9B signal in neurons colocalising with TH (SNc, control vs 48 h, 100 ± 24 vs 226 ± 28 , $p < 0.01$ and VTA control vs 48 h, 100 ± 18 vs 222 ± 21 , $p < 0.01$) (Fig. 4C).

For a more precise quantification of RAD9B protein levels, Western-blot experiments were performed under the same conditions as immunohistochemistry (Fig. 3D). Thus, we observed once more the increase in RAD9B protein levels (control vs 48 h, 1 ± 0.12 vs 2.2 ± 0.25 , $p < 0.01$) in the midbrain of rats sacrificed 48 h after

6-OHDA treatment and the protein level recovery in those sacrificed after 5 days (Fig. 3E) compared to controls. A TH antibody was used as a control to ensure that the same number of midbrain DAergic neurons were used in the study. At the same time, an actin antibody was used to control the level of degeneration of DAergic neurons. Although mild degeneration is known to exist at the SNcv level, no differences were observed in the levels of degeneration between controls and 6-OHDA injected rats in the total midbrain extract using Western-blot.

4. Discussion

The mesostriatal system plays a critical role in the regulation of motor function and reward processes, making it a key target for the study of neurodegenerative disorders such as Parkinson's disease (PD). One interesting checkpoint involved in maintaining genomic integrity is RAD9B, which has been implicated in various cellular processes, including DNA damage repair and cell cycle control. Despite its potential significance, the expression pattern and functional relevance of RAD9B in the mesostriatal system, particularly in the context of PD pathology, remain largely unexplored. Furthermore, the limited number of studies investigating the role of RAD9B in different systems or pathologies highlights the need for further investigation to elucidate its precise contributions. In this study, we present novel findings on the expression of the RAD9B

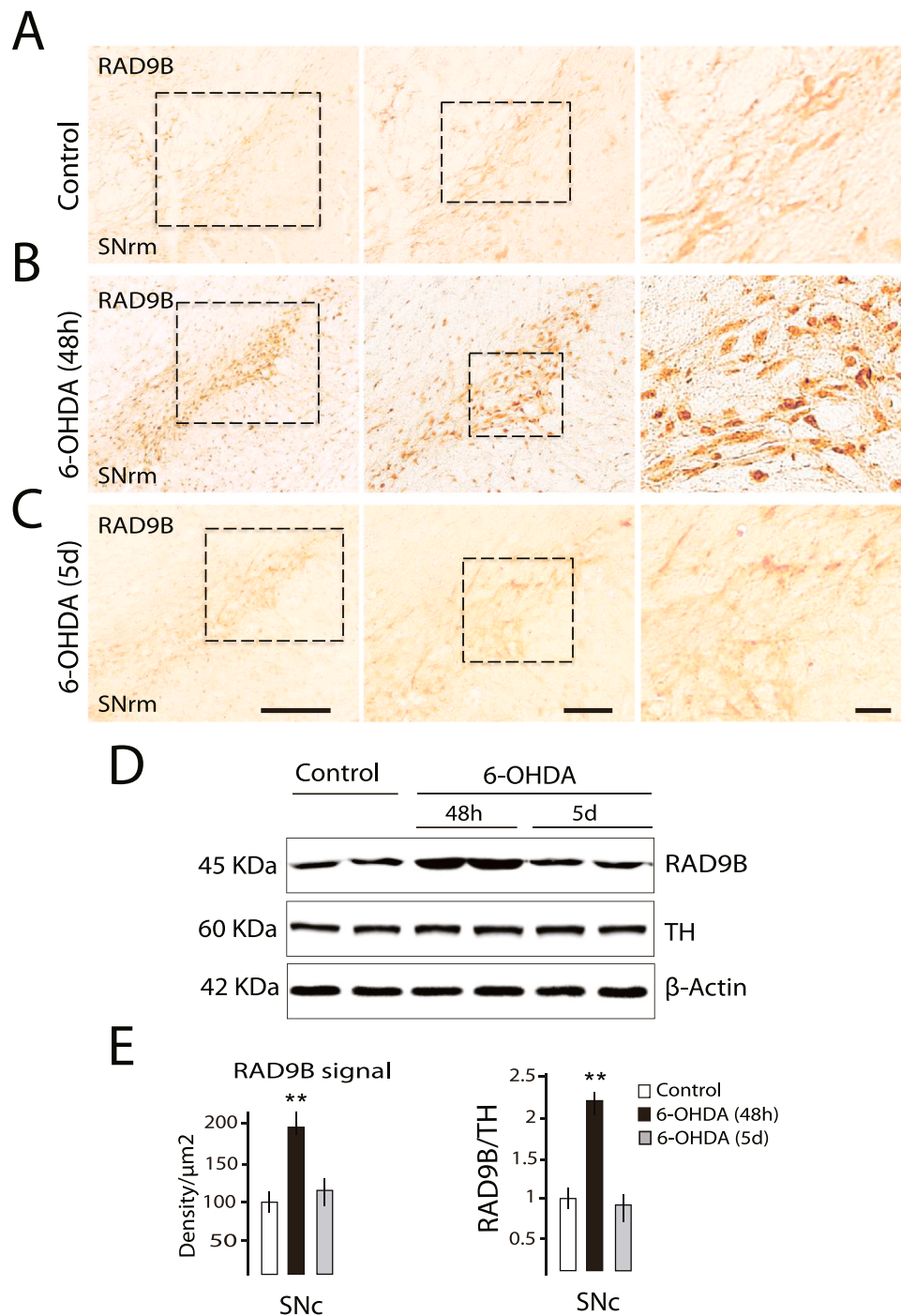


Fig. 3. A-C, RAD9B immunohistochemistry staining in rat midbrain in the 6-OHDA model. A, RAD9B expression in control rats (treated with vehicle). B, RAD9B expression in rats treated with 150 µg of 6-OHDA and sacrificed 48 h after injury. C, RAD9B expression in rats treated with 150 µg of 6-OHDA and sacrificed 5 d after injury. The dashed box represents the magnified area for densitometric analysis of RAD9B-positive neurons (right column). D, RAD9B expression by Western-blot in control and 6-OHDA-injured rats sacrificed 48 h or 5 d after injury. To determine the number of DAergic neurons and the degree of degeneration, anti-TH and anti-β-Actin antibodies were used, respectively. E, Densitometric quantification of RAD9B immunohistochemistry (left column) and Western-blot (right column). ** indicates $p < 0.01$. Bars, mean $\pm$ SEM. SNrm; substantia nigra rostro-medial; SNc: substantia nigra pars compacta. Scale bars: 300, 100 and 50 µm respectively.

checkpoint in both rat and human mesostriatal systems. In addition, we investigate the altered expression of RAD9B in dopaminergic mesencephalic neurons of a rat model subjected to the neurotoxin 6-OHDA, which mimics the degenerative process observed in PD. By addressing this knowledge gap, our study aims to shed light on the potential involvement of RAD9B in PD.

In adult humans, previous studies have reported the expression of RAD9B mRNA in testes and skeletal tissues, while in mice, it can be detected in the brain, liver and mammary glands (Dufault et al.,

2003). However, these findings also suggested that the detection methods used may not have been sensitive enough to prove RAD9B expression in other regions. In this work, we have demonstrated the existence of RAD9B expression in both rat and human midbrain. To achieve this, we used an anti-RAD9B antibody that has been previously tested and published (Pérez-Castro and Freire, 2012). In addition to IHC, immunofluorescence and Western-blot analysis, we implemented qPCR and ISH techniques. The combined results from these techniques demonstrated the existence of RAD9B expression

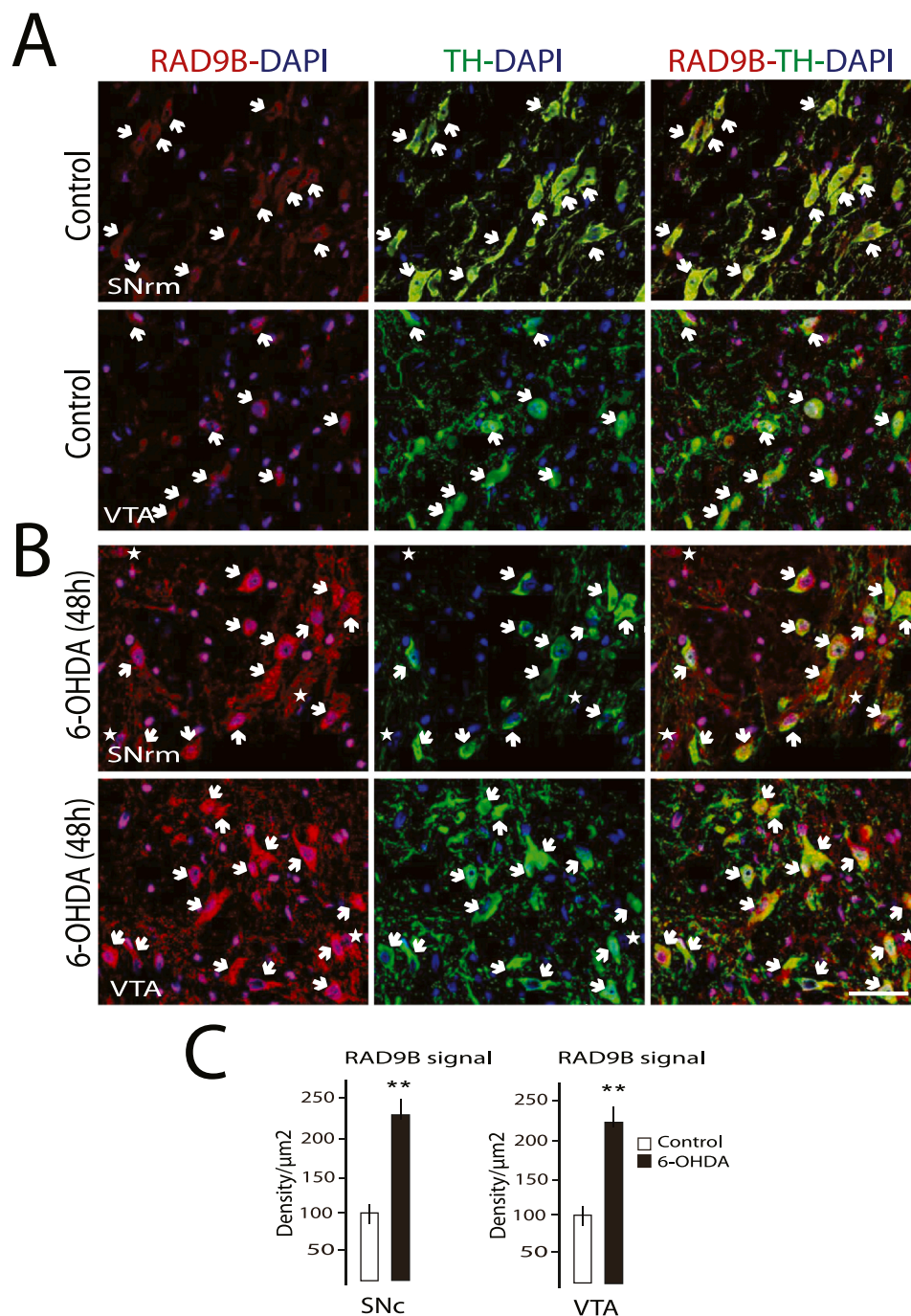


Fig. 4. A-B, Representative immunofluorescence co-staining for RAD9B and TH in rat SNrm and VTA treated with vehicle (A) or 150 µg of 6-OHDA (B) and sacrificed 48 h after injection. White arrows indicate areas of RAD9B and TH co-localisation and white stars indicate no co-localisation. C, Densitometric quantification of immunofluorescence staining for RAD9B in TH-positive neurons in the SNc (left column) and VTA (right column). Although only the SNrm region is shown, the analysis has been performed on the entire SNc. C, Densitometric analysis of immunofluorescence for RAD9B in TH-positive neurons in the SNc and VTA. ** indicates p < 0.01. Bars, mean ± SEM. SNrm: rostro-medial substantia nigra; VTA: ventral tegmental area; SNc: substantia nigra pars compacta. Scale bars: 20 µm.

at both the gene and protein levels in the midbrain of rats. Notably, we observed specific expression of RAD9B in mesencephalic DAergic neurons of both rat and human samples. This finding holds significant relevance as these DAergic neurons are directly affected in PD and contributes to a better understanding of the underlying pathologic mechanisms.

Various models have been developed utilizing 6-OHDA neurotoxin to induce a degenerative process resembling PD in rats (Blesa et al., 2012; Bové et al., 2012; Simola et al., 2007; Guimarães et al., 2021). Traditionally, unilateral lesions have been created by injecting 6-OHDA into the SN, medial forebrain bundle or striatum (Jeon,

1995; Blandini et al., 2007). However, in this study, we have used a model in which 6-OHDA was administered in the third ventricle, resulting in bilateral progressive degeneration within the mesostriatal system. This model closely replicates morphological and behavioural characteristics of those found in PD (Rodríguez et al., 2001). In our study, we injected a specific dose of 150 µg of 6-OHDA to induce a target lesion which resulted in a minor degeneration in the SNcv and no degeneration in the SNrm, suggesting a potential protective effect on DAergic neurons. Within 48 h of 150 µg 6-OHDA neurotoxin injection, SN neurons exhibit a regulatory response by modulating the expression of DAT; decreasing glycosylated DAT and

increasing non-glycosylated DAT protein levels (Afonso-Oramas et al., 2010). At 48 h after injury, the decrease in glycosylated DAT reduces the ability of the DAergic neuron to facilitate the entry of 6-OHDA through the plasma membrane, which would be protecting the cell from the stress produced by the toxin. We observed an increase of RAD9B both at the genomic and protein level in midbrain neurons 48 h after toxin injection. Specifically, the overexpression of RAD9B protein was observed to be localised in midbrain DAergic neurons. We have focused our investigation on the protein levels of RAD9B within the mesostriatal system due to its potential functional role in the most susceptible region in PD. The observed upregulation of RAD9B protein in response to neurotoxin-specific damage to DAergic neurons aligns with the established function of the RAD9 family of checkpoints in preserving genomic integrity (Lancelot et al., 2007; Grenon et al., 2007; Canfield et al., 2009). Similar activation of these DNA damage response mechanisms has been reported in both familial and idiopathic forms of PD (Martin, 2008; Gonzalez-Hunt and Sanders, 2021; Li et al., 2022). Furthermore, RAD9 family proteins and 9–1–1 heterotrimer also play an important role in apoptotic processes (Broustas and Lieberman, 2012; Helt et al., 2005). Given that exposure to 6-OHDA initiates an apoptotic cascade through DNA damage induced by reactive oxygen species (ROS) (Bernstein et al., 2011; Shukla et al., 2014), the involvement of RAD9B in this context is particularly relevant.

In this study, we also observed an increase in mRNA expression of RAD9B and RAD1, but not HUS1, in response to the neurotoxin. Although there was a subtle trend towards increased HUS1 expression, this did not reach statistical significance. While the primary focus of this work was to examine RAD9B expression, it would be valuable in future studies to examine the protein levels of RAD1 and HUS1. Such investigations would provide insight into their functionality within this system and further enhance our understanding of the RAD9 family of checkpoints in the context of PD pathology.

This study used a PD model in which RAD9B overexpression was observed 48 h after exposure to 150 µg 6-OHDA. This overexpression returned to normal levels within 5 days, and no significant neuronal degeneration was detected. These results suggest that the role of RAD9B may depend on the severity of oxidative damage produced, and depending on the viability of the affected cell, RAD9B could potentially have a reparative and protective function or trigger an apoptotic cell death response.

Our study provides a framework for future investigations into the role of RAD9B in the differential vulnerability of the mesostriatal system. It also provides a basis for further studies into the behaviour of this checkpoint in the selective degeneration of midbrain DAergic cells and its implication as a target in the development or delay of the degenerative course of PD. By further exploring these aspects, we can gain valuable scientific insight into the underlying mechanisms of PD and investigate potential therapeutic approaches.

Ethical Statement

Human brains were obtained according to national Spanish guidelines and under the supervision of the Ethics Committee of the University of La Laguna, in accordance with the Declaration of Helsinki, 1975. Experimental protocols in rats were approved by the Ethics Committees of the University of La Laguna under the European Directive of 22 September 2010 (2010/63/EU) and the ARRIVE guidelines.

CRediT authorship contribution statement

DAO and PBCH designed the study. DAO, PBCH and JSH performed the experiments. ICM and JLF analysed the data. DAO wrote the article. PBCH and RF provided useful comments and feedback on

the draughting of this manuscript. All authors participated in and approved the final version of the manuscript.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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