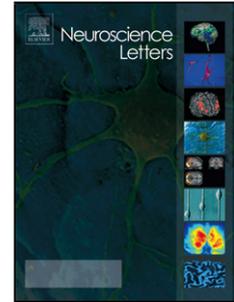


Accepted Manuscript

Title: A role for DJ-1 against oxidative stress in the mammalian retina

Authors: José Martín-Nieto, Mary Luz Uribe, Julián Esteve-Rudd, María Trinidad Herrero, Laura Campello



PII: S0304-3940(19)30464-1
DOI: <https://doi.org/10.1016/j.neulet.2019.134361>
Article Number: 134361

Reference: NSL 134361

To appear in: *Neuroscience Letters*

Received date: 3 May 2019
Revised date: 24 June 2019
Accepted date: 1 July 2019

Please cite this article as: Martín-Nieto J, Luz Uribe M, Esteve-Rudd J, Trinidad Herrero M, Campello L, A role for DJ-1 against oxidative stress in the mammalian retina, *Neuroscience Letters* (2019), <https://doi.org/10.1016/j.neulet.2019.134361>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

A role for DJ-1 against oxidative stress in the mammalian retina

José Martín-Nieto^{a,b,*}, Mary Luz Uribe^{a,1}, Julián Esteve-Rudd^{a,2}, María Trinidad Herrero^c and Laura Campello^{a,3}

^a. Departamento de Fisiología, Genética y Microbiología, Facultad de Ciencias, Universidad de Alicante, 03080 Alicante, Spain

^b. Instituto Multidisciplinar para el Estudio del Medio “Ramón Margalef” (IMEM), Universidad de Alicante, 03080 Alicante, Spain

^c. Neurociencia Clínica y Experimental (NiCE), Facultad de Medicina, Instituto de Investigación en Envejecimiento, Instituto Murciano de Investigación Biosanitaria (IMIB), Universidad de Murcia, 30071 Murcia, Spain

* Corresponding author at: Departamento de Fisiología, Genética y Microbiología, Facultad de Ciencias, Campus Universitario San Vicente, Universidad de Alicante, 03080 Alicante, Spain.

E-mail address: jmnieto@ua.es (J. Martín-Nieto)

¹. Present address: Department of Biological Regulation, Faculty of Biology, Weizmann Institute of Science, 76100 Rehovot, Israel

². Present address: Bayer AG, 42096 Wuppertal, Germany

³. Present address: Neurobiology-Neurodegeneration and Repair Laboratory, National Eye Institute (NEI), National Institutes of Health, Bethesda, MD 20892, USA

Bullet points

- DJ-1 mRNA and protein are expressed in the neural retina and RPE across mammals
- DJ-1 and α -synuclein are upregulated in the retina of rotenone-treated rats

- Elevated levels of oxidized DJ-1 are found in the parkinsonian rat retina

DJ-1 likely plays in the retina a major role in neuroprotection from oxidative stress

ABSTRACT

We have previously reported the expression of Parkinson disease-associated genes encoding α -synuclein, parkin and UCH-L1 in the retina across mammals. DJ-1, or parkinsonism-associated deglycase, is a redox-sensitive protein with putative roles in cellular protection against oxidative stress, among a variety of functions, acting through distinct pathways and mechanisms in a wide variety of tissues. Its function in counteracting oxidative stress in the retina, as it occurs in Parkinson and other human neurodegenerative diseases, is, however, poorly understood. In the present study, we address the expression of DJ-1 in the mammalian retina and its putative neuroprotective role in this tissue in a well-known model of parkinsonism, the rotenone-treated rat. As a result, we demonstrate that the *DJ1* gene is expressed at both mRNA and protein levels in the neural retina and retinal pigment epithelium (RPE) of all mammalian species studied. We also present evidence that DJ-1 functions in the retina as a sensor of cellular redox homeostasis, which reacts to oxidative stress by increasing its intracellular levels and additionally becoming oxidized. Levels of α -synuclein also became upregulated, although parkin and UCH-L1 expression remained unchanged. It is inferred that DJ-1 likely exerts in the retina a potential neuroprotective role against oxidative stress, including α -synuclein oxidation and aggregation, which should be operative under both physiological and pathological conditions.

Keywords: Parkinson disease, DJ-1, retina, gene expression, oxidative stress, rotenone

1. INTRODUCTION

The *DJ1* gene is found in the human locus PARK7 (OMIM 602533), located in chromosomal region 1p36 and originally identified as a novel proto-oncogene [1]. Its encoded product is a highly conserved protein, known as parkinsonism-associated deglycase and recently renamed as protein/nucleic acid deglycase DJ-1 (UniProtKB Q99497), composed of 189 amino acids and belonging to the DJ-1/ThiJ/Pfpl superfamily [2]. DJ-1 is a multifunctional, redox-sensitive protein with putative roles in cell protection against oxidative stress, mitochondrial physiology, transcriptional regulation, modulation of proteasome activity and as a redox-sensitive molecular chaperone, acting through distinct pathways and mechanisms presumably in a wide variety of tissues and cell types [3]. Mutations in the *DJ1* gene are causative of autosomal recessive early-onset Parkinson disease (PD) (OMIM 606324), although they are also associated with the pathogenic mechanisms of sporadic PD and Alzheimer (AD) diseases [4,5], as well as of other brain and ocular neurodegenerative disorders where oxidative stress is implicated [6,7].

DJ-1 is ubiquitously present in many organs, including the brain, heart, skeletal muscle, liver, pancreas, kidney and placenta [1,8]. In the brain DJ-1 is widely distributed, being highly expressed in the substantia nigra and striatum, two areas critically involved in PD pathogenesis, amongst other brain regions [2,8]. In the brain of PD and AD patients DJ-1 is found both upregulated and oxidized, experiencing an acidic shift in its isoelectric point (pI) that is crucial to exert its neuroprotective functions [5,6].

A growing body of experimental evidence has accumulated concerning visual dysfunction in the retina of parkinsonian patients, including loss of visual acuity, contrast sensitivity, color discrimination and motion perception [9,10]. These PD-associated symptoms have been correlated to morphological, electrophysiological and psychophysical impairments in retinal structure and function, in addition to disorders of cortical visual processing [9,10]. However, oculo-visual features specifically associated with particular *DJ1* mutations have not been reported in PD patients. By contrast, a series of significant

molecular, structural and functional abnormalities have been recently described in the retina of *Dj1* KO mice [11]. Despite DJ-1 has been extensively studied in brain neurons, its role in the neural retina is poorly understood. In the present study, we address the expression of the *DJ1* gene at the mRNA and protein levels in the neural retina and retinal pigment epithelium (RPE) of a variety of mammals. As well, we assess its presumptive neuroprotective role in the retina of a well-known model of parkinsonism, the rotenone-treated rat, by analyzing possible changes in the expression levels and oxidation status of DJ-1. Alongside, given that functional relationships have been described between DJ-1 and other PD-related proteins [12,13], we have also addressed possible alterations in the expression of α -synuclein, parkin and UCH-L1, whose expression we have previously evidenced in the normal mammalian retina [14,15].

2. MATERIALS AND METHODS

2.1. Biological material

In this work samples from adult mammalian species were analyzed, including mouse (*Mus musculus*, C57BL/6J), rat (*Rattus norvegicus*, Sprague-Dawley), cow (*Bos taurus*) and cynomolgus monkey (*Macaca fascicularis*). All animal handling was performed in compliance with the rules set by the National Institutes of Health (USA), the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the European Directive 2010/63/EU. Rodents were held at the Universidad de Alicante animal care facility and, after being anesthetized and euthanized according to well-standardized, internationally-approved protocols, their eyeballs were enucleated and stored in RNA*later* solution (Ambion; Austin, TX, USA). Bovine eyes were freshly obtained from a local slaughterhouse. Monkeys were kept as indicated [16] at the animal care facility of the Universidad de Murcia (Spain) in accordance with guidelines given by the International Primate Society. All protocols and primate handling procedures used were approved by the university bioethics research committee, and their eyeballs were immediately enucleated following anesthesia and sacrifice, as described [16]. Bovine and monkey eyes were snap-frozen in liquid N₂ and kept at –80 °C until the retinas were dissected for RNA or protein isolation. RNA from human retina was purchased from Clontech BD (Mountain View, CA, USA).

For rotenone treatment, rats weighing ca. 300 g and aged 2-3 months at the beginning of the experiment were divided into two groups, one of which was injected subcutaneously with rotenone and the other kept as control, as described [17]. One week after the last administration of rotenone, video recordings of the rats from both groups were performed in order to evaluate motor impairments [17].

2.2. Conventional and quantitative RT-PCR

Eyes were thawed and the retina was obtained free from other ocular tissues and dissected into RPE and neural retina. RNA was extracted using the TRIzol reagent from

Invitrogen (Carlsbad, CA, USA) and further treated with DNase I (Ambion; Austin, TX, USA). Reverse transcription to cDNA and conventional PCR amplification were performed essentially as described [14,15] using the species-specific *DJ1* primers indicated in **Table S1** (see Appendix A). Real-time PCR was performed using the qPCR FastStart SYBR Green reaction mix (Roche; Barcelona, Spain) and primers specific for the rat *Dj1*, *Snca*, *Th* and *Gapdh* genes enlisted in **Table S1**, as indicated [17]. The fluorescence increase during PCR was monitored on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems; Foster City, CA, USA).

2.3. Western blotting

Retinal proteins were extracted and subjected to immunoblotting analysis as previously described [14,15,17]. Proteins (50 µg/lane) were resolved by SDS-PAGE on 5-20% polyacrylamide-gradient gels and electrotransferred to Hybond-P PVDF membranes (GE Healthcare, Buckinghamshire, UK). These were then probed at 4 °C overnight with primary antibodies to human proteins enlisted in **Table S2** (see Appendix A) at the dilutions indicated, and thereafter incubated at room temperature for 1 h with horseradish peroxidase (HRPO)-conjugated secondary antibodies to IgG at a 1:10,000 dilution. Detection was performed by enhanced chemiluminescence (ECL) using the SuperSignal West Dura system (Pierce) followed by Hyperfilm ECL film (GE Healthcare) exposure to blots.

2.4. 2D Gel electrophoresis and OxyBlot analysis

For the analysis of oxidative modification of proteins by carbonylation, samples were prepared as described [18,19] and thereafter analyzed using the OxyBlot Protein Oxidation Detection kit (Merck Millipore; Darmstadt, Germany). Accordingly, proteins (300 µg) extracted from neural retinas were precipitated with trichloroacetic acid on ice and then derivatized with the 2,4-dinitrophenylhydrazine (DNPH) solution provided in the kit. After stopping the reaction, the pellets were washed with ethanol and then dissolved in rehydration solution. For isoelectric focusing (IEF) in the first dimension, the samples were loaded onto Immobiline DryStrips (linear pH 3-10 gradient, IPG strips) from GE Healthcare

(Buckinghamshire, UK), and IEF was performed as previously described [16]. Then the strips were incubated in equilibration buffer, and electrophoresis in the second dimension was carried on 12.5% SDS-PAGE gels [16]. Proteins were then electrotransferred to PVDF membranes, and subsequently probed at 4°C overnight with the rabbit antibody to dinitrophenyl (DNP) groups provided in the kit at a 1:150 dilution. Thereafter, they were incubated with HRPO-conjugated goat anti-rabbit IgG, and detection was performed by ECL. Subsequently, the blot was stripped of antibodies and reprobed with rabbit polyclonal antibody to DJ-1 (ab12857; Abcam, UK) at a 1:5,000 dilution. Densitometric quantitation of protein spots recorded on X-ray films from control and rotenone-treated rats was accomplished using the NIH ImageJ software.

2.5. Statistical analyses

For qRT-PCR, reactions were carried out in triplicate, and 3-5 animals per group (control and rotenone-treated rats) were analyzed. Results were evaluated by the $\Delta\Delta C_t$ method, and are presented as the ratio between mRNA levels of the gene of interest to mRNA levels of an internal housekeeping gene, *Gapdh*. Protein band intensities from Western blots were quantified using the NIH ImageJ software, and normalized to β -actin levels. Differences between control and rotenone-treated rat retinas were contrasted using a non-parametric, two-tailed *t*-Student test, with a confidence interval of 0.95, which was applied using the GraphPad software (Prism; La Jolla, CA, USA).

3. RESULTS

Expression in retinal tissue of the *DJ1* gene or its protein product has only been previously addressed in a limited number of species. In order to undertake a more systematic study across mammals, expression of the *DJ1* gene was analyzed first at the mRNA level separately in the RPE and neural retinal fractions from rodents (mouse and rat), cows and primates (monkey and human). As shown in **Fig. 1A**, its encoded mRNA was found by means of RT-PCR analysis to be present in the neural retina of all mammals studied. Additionally, expression of this gene was detected in the mouse, rat, bovine and monkey RPE. All PCR products obtained exhibited the expected size, and were verified by automated DNA sequencing. Negative RT-PCR controls showed no amplification (data not shown). Next, expression of the DJ-1 protein was evaluated by Western blotting. As shown in **Fig. 1B**, DJ-1 was detected in rodent (mouse and rat), bovine and monkey neural retinas. This analysis also showed the presence of this protein in the RPE of all the species studied. In all cases a single band of the expected molecular mass (M_r), 23 kDa, was obtained (**Fig. 1B**), which was not detected when omitting the primary antibody to DJ-1 (data not shown).

Rats treated with rotenone constitute an established animal model of parkinsonism, exhibiting both PD-associated brain [20,21] and retinal alterations [17,22,23]. Therefore, in order to analyze possible alterations in the expression of PD-related genes, including *DJ1*, under parkinsonism conditions, we treated rats systemically with this pesticide in order to induce a stable parkinsonian syndrome. In our hands, rotenone-treated rats exhibited a series of noticeable motor deficiencies, such as rotatory movements, postural disturbances and balance alterations [17,24]. In the retina, we found that these rats underwent a loss of the dopaminergic (DA) cell marker, tyrosine hydroxylase (TH), detected at both mRNA and protein levels ([17] and this work, data not shown). This decrease of TH expression was in keeping with a reduction of the extent of the DA plexus and number of cell bodies in the inner retina, together with a loss of photoreceptors and

a reduced synaptic connectivity in the outer retina between those remaining and their postsynaptic neurons [17].

Expression of genes encoding α -synuclein, parkin and UCH-L1 has been previously reported by our group in the normal rat retina [14,15]. In this work we first evaluated the expression of the α -synuclein gene (*Snc α*) at the mRNA and protein levels in the neural retina of parkinsonian rats in comparison to control individuals (**Fig. 2A-C**). We found a significant increase, of 2.27 ± 0.53 fold, in the levels of α -synuclein protein in the retina of rats treated with rotenone, as compared to control rats (**Fig. 2A,B**). This change was in agreement with the results obtained in qRT-PCR assays, where we observed a small, yet significant increase in the mRNA levels of the *Snc α* gene, of 1.54 ± 0.11 fold relative to those of untreated rats (**Fig. 2C**). Next, the expression of the *Dj1* gene was quantified at the mRNA and protein levels in the retina of these animals (**Fig. 2F-H**). It was found that levels of the DJ-1 oxidative stress-sensing protein were greatly increased in the parkinsonian rats, by 2.92 ± 0.66 fold (**Fig. 2F,G**). However, no significant change was observed in the levels of the *Dj1* mRNA in the retina of rotenone-treated rats (**Fig. 2H**). Additionally, we evaluated the levels of expression of parkin and UCH-L1, two proteins previously associated with PD which participate in the ubiquitin-proteasome system (UPS). We did not observe statistically-significant changes in the levels of either parkin (**Fig. 2D,E**) or UCH-L1 (**Fig. 2I,J**) in the retina of parkinsonian rats by Western blotting, allowing to conclude that the damage induced by rotenone in the rat retina did not affect the expression of these two proteins.

Under oxidative stress conditions, a decrease in the pI of DJ-1 (acidification) generally occurs, attributable to its oxidation, which is crucial for this protein to exert its neuroprotective functions [4,25,26]. In this work we assessed the possible oxidation of DJ-1 protein via carbonylation in the neural retina of rotenone-treated rats. With this purpose, we used a well-established OxyBlot analysis aimed at detecting protein carbonyl groups upon their derivatization with DNPH, followed by immunodetection with an antibody to resulting DNP groups. Accordingly, retinal proteins extracted from rotenone-

treated and control rats were subjected to derivatization with DNPH, separated by 2D gel electrophoresis, and then analyzed by Western blotting using an anti-DNP antibody. In order to identify the exact location(s) of DJ-1 protein in our 2D blots, antibody stripping was performed followed by reprobing with the antibody to DJ-1 used above. As shown in **Figure 3A**, the DJ-1 antibody specifically recognized a series of four protein spots in a row, all of them exhibiting the same apparent Mr (~23 kDa) but with different isoelectric points (pI 5.4, 5.6, 5.9 and 6.3). This evidenced the presence of (at least) four DJ-1 protein isoforms in the rotenone-treated rat retina (**Fig. 3A**, second panel), of which the most acidic one (pI 5.4) was not detected in the untreated-rat retina (**Fig. 3A**, first panel). Out of the four DJ-1 spots we detected in parkinsonian rats, only that with the highest pI (6.3) was immunoreactive to the DNP antibody, indicating that only this isoform was oxidatively modified by carbonylation (**Fig. 3A**, third panel). The lack of detection of carbonyl groups in the other three, more acidic forms of this protein (pI 5.4 to 5.9), could reflect that they represented non-oxidized forms of DJ-1, as expected [5], or else be attributable to their lower abundance compared to the pI 6.3 isoform. It was also found that a dramatic increase in the total levels of the oxidized, 23 kDa/pI 6.3 DJ-1 isoform, occurred in the retinas of parkinsonian rats (**Fig. 3A**, fourth panel) as compared to control, untreated animals (**Fig. 3A**, third panel). In fact, quantitation by densitometry of immunoreactivity to the DNP antibody revealed a statistically significant increase, of 2.71 ± 0.19 fold, in the levels of oxidized DJ-1 protein in the retinas of rats treated with rotenone (**Fig. 3B**).

4. DISCUSSION

We have previously reported expression of PD-associated genes encoding α -synuclein, parkin and UCH-L1 (namely *SNCA*, *PARK2/PRKN* and *UCHL1*) at both mRNA and protein levels in the neural retina and RPE of a number of mammalian species [14,15]. In this work, we have evidenced the presence of the *DJ1* mRNA and its encoded protein in both the neural retina and RPE tissue of all mammals analyzed, including rodents (mouse and rat), cows and primates (monkey and human). This adds up to a previous report where expression of the *DJ1* gene mRNA was reported in the rat and marmoset retinas [27]. By Western blotting we detected a single, 23 kDa band in all cases, whose Mr was compatible with that previously reported in rodent and marmoset retinal fractions [11,27] and RPE cell lines [28]. Taken together, these results provide evidence that expression of the DJ-1 encoding gene at the mRNA and protein levels is widespread across mammals, and occurs both in the RPE and neural portions of the retina. A coherent DJ-1 expression pattern has only been shown by immunohistochemistry in the rodent (mouse and rat) retina, where it is mainly found in the RPE and photoreceptor cells, mostly at their inner segments (where mitochondria are located) and with lower levels in the outer plexiform layer (OPL) [11,29].

In this work, we have observed an upregulation of α -synuclein and DJ-1 expression in the retina of rats treated with rotenone, a natural compound known to act as a potent inhibitor of mitochondrial complex I [12]. These alterations sum to our previously-reported structural and functional impairments related to parkinsonism in rats, including a series of motor anomalies together with morphological alterations in photoreceptors and their synaptic connectivity in the retina, and deficiencies in electroretinogram (ERG) recordings [17,24]. At the molecular level, these rats exhibited as well a loss of TH expression (mRNA, protein and retinal immunoreactivity) associated with a reduction of the DA plexus and density of cell bodies [17], in keeping with a previous study on rotenone-treated rats [22] and in a similar fashion as we had reported in parkinsonian

monkeys, treated with the neurotoxic inducer of parkinsonism, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [30].

To our knowledge, the present study is the first evidencing alterations in the expression of α -synuclein in the retina of a PD animal model. The increase we detected in the levels of the *Snc α* mRNA was in accordance with previous reports in the midbrain of MPTP-treated mice [31] and idiopathic PD humans [32], where upregulated levels of the α -synuclein encoding mRNA are generally found. The elevation of α -synuclein protein we observed in the retina of our parkinsonian rats was also in agreement with this protein being consistently overexpressed in the substantia nigra of rodents treated with rotenone [33] or MPTP [34,35], and human PD patients [35]. This α -synuclein increase has been proposed to be epigenetically regulated in brain neurons [34] and part of a neuronal response to oxidative stress involving α -synuclein elevation, DJ-1 oxidation (see later in Discussion) and UPS dysfunction, albeit eventually with deleterious consequences [12,21,36]. In this context, oxidative stress-promoted conformational changes in α -synuclein [37,38], together with its elevated levels and UPS impairment would result in aggregation of misfolded α -synuclein, this leading to degeneration and/or death of cells especially vulnerable to oxidative stress, such as DA neurons in the brain [39], and both photoreceptors and DA cells in the retina [17,22].

We did not observe alterations in the levels of parkin or UCH-L1 in the retina of rats treated with rotenone. In the case of parkin, alterations in *Park2* mRNA expression have not been found in the substantia nigra of mice treated with MPTP [40], although mRNA and/or parkin levels were reportedly lower in patients with idiopathic PD [41] and in rotenone-treated rats [42]. Regarding UCH-L1, decreased expression has been detected at both mRNA and protein levels in the brain of patients with idiopathic PD [43], although levels of this protein have also been found unchanged in the substantia nigra of PD patients [44]. It follows that, whereas parkin and UCH-L1 do not appear to be affected by rotenone-promoted oxidative stress in the retina, or have a role in its counteraction, the situation may be different in the brain of PD humans and rodents.

Regarding DJ-1 protein, we have found in this work an increase in the retina of rats treated with rotenone, although we did not detect the corresponding change in the levels of its encoding mRNA. This may reflect that regulation of DJ-1 protein expression occurs post-transcriptionally, for instance by increasing the *Dj1* mRNA half-life and/or its translation rate in the cytoplasm. An elevation of DJ-1 protein levels in the human PD brain [5,6] and in the midbrain of rotenone-treated mice [45] has been reported, in keeping with our results in the rat retina. It is well known that DJ-1 acts, as its main function, as an antioxidant scavenger and redox-sensitive protein protecting neuronal cells against oxidative stress in a variety of human brain and ocular neurodegenerative diseases [6,7]. Therefore, an increase in its expression levels in the retina likely reflects a potential neuroprotective cellular response to the increase in reactive oxygen species (ROS) induced by the inhibitory effect of rotenone on mitochondrial electron transport, as demonstrated in cultured neurons [46,47]. An increase of DJ-1 expression has also been documented to occur upon aging in the RPE from rats and marmosets [27,29], as well as of human age-related macular degeneration (AMD) patients [28]. Yet, how this protein exerts its neuronal protective role is not clear, and in this light DJ-1 has been shown to interact with α -synuclein by virtue of its redox-sensitive chaperone activity, in an attempt to prevent its oxidation-dependent aggregation and toxicity [12,48,49]. Also, DJ-1 is thought to work in parallel with parkin and PINK1 by forming a ubiquitin E3 ligase complex in order to maintain mitochondrial function in response to rotenone [50], and in this context DJ-1 has also been reported to translocate into the mitochondria in cultured mouse RPE cells under oxidative stress [28].

Additionally, by using a combination of 2D gel electrophoresis and Western blotting analysis, we have identified four isoforms of DJ-1 protein with different pI values in the rat retina. The presence of multiple pI isoforms of DJ-1 has also been reported in the normal human brain, as well as in PD and AD patients [4–6]. The human and rat DJ-1 proteins contain three and four cysteine residues, respectively, of which Cys-106 is unusually reactive and becomes preferentially oxidized under oxidative stress [3,6,25],

thereby playing a critical role in DJ-1 neuroprotective activities, including its chaperone activity towards α -synuclein [48]. This is coherent with the fact of DJ-1 oxidized at Cys-106 being found in DA neuronal cell bodies and astrocytes in the human substantia nigra and striatum [3,26]. In the present study, we also report the presence of elevated levels of oxidized DJ-1 protein in the rat neural retina, in response to oxidative stress induced by treatment with rotenone. Only the pI 6.3 isoform was found to increase in the retina of our parkinsonian rats, in keeping with results in human PD and AD brains [5] and in RPE lysates from donors with AMD [28], showing that this is the only oxidatively-modified DJ-1 isoform increasing in these disorders by using our same methodology.

The importance of DJ-1 for proper retinal structure and function has been recently stressed out by a series of structural and physiological analyses of the retina of *Dj1* KO mice. As reported, these animals displayed a significant reduction in overall retinal thickness, which was prominent in the RPE and photoreceptor layers including the OPL, i.e., in retinal cells known to express DJ-1 [11,49]. As well, *Dj1* KO mice exhibited disorganized outer segments with decreased expression of opsins, and degeneration of DA amacrine cells. Interestingly, many of these impairments were in common to our parkinsonian rats, including a dramatic loss of mitochondria in the inner segments and axon terminals of photoreceptors [17]. Furthermore, at the physiological level ERG alterations in the b-wave were also detected in both rodent models of PD [11,13,49].

5. CONCLUSIONS

Collectively, results reported in this work together with previous findings are indicative that DJ-1 potentially plays a major role in protecting retinal neurons and RPE cells from oxidative damage occurring naturally (i.e. during aging), induced by treatment with oxidizing agents, or under pathological conditions (such as PD, AD or AMD). DJ-1 would thus function in the retina as a sensor of cellular redox homeostasis, reacting to oxidative stress by increasing its expression levels and oxidation status. Yet, the retinal changes reported here in the rotenone-treated rat model need to be corroborated in other

mammalian models of PD, in order to further shed light on their relationship with ocular symptoms associated with this disease.

AUTHORS CONTRIBUTIONS

MLU, JE-R and LC conducted the experiments and together with JM-N interpreted the results. Monkey eyes were provided by MTH. Funding was obtained by JM-N and MTH. JM-N wrote the paper, which was revised and finally approved by all authors.

Declarations of interest: None

ACKNOWLEDGMENTS AND FUNDING

This research was supported by grants from the Instituto de Salud Carlos III (PI09/1623 to JM-N, and PI10/02827 and PI13/01293 to MTH), cofinanced by the European Regional Development Fund (ERDF/FEDER), and from Fundación Séneca (19540/PI/14 to MTH). Additional funding was awarded by the Universidad de Alicante (UA) for use of technical research facilities (ref. UAUSTI18-16), diffusion of research results (ref. UADIF 18-52) and scientific productivity (ref. VIGROB-237). MLU, JE-R and LC were recipients of predoctoral contracts/fellowships from the UA. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

REFERENCES

- [1] D. Nagakubo, T. Taira, H. Kitaura, M. Ikeda, K. Tamai, S.M.M. Iguchi-Ariga, H. Ariga, DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with *ras*, *Biochem. Biophys. Res. Commun.* 231 (1997) 509–513.
- [2] V. Bonifati, P. Rizzu, M.J. Van Baren, O. Schaap, G.J. Breedveld, E. Krieger, M.C.J. Dekker, F. Squitieri, P. Ibanez, M. Joesse, J.W. Van Dongen, N. Vanacore, J.C. Van Swieten, A. Brice, G. Meco, C.M. Van Duijn, B.A. Oostra, P. Heutink, Mutations in the *DJ-1* gene associated with autosomal recessive early-onset parkinsonism, *Science*. 299 (2003) 256–259.
- [3] Y. Saito, DJ-1 as a biomarker of Parkinson's disease, *Adv. Exp. Med. Biol.* 1037 (2017) 149–171.
- [4] R. Bandopadhyay, A.E. Kingsbury, M.R. Cookson, A.R. Reid, I.M. Evans, A.D. Hope, A.M. Pittman, T. Lashley, R. Canet-Aviles, D.W. Miller, C. McLendon, C. Strand, A.J. Leonard, P.M. Abou-Sleiman, D.G. Healy, H. Ariga, N.W. Wood, R. De Silva, T. Revesz, J.A. Hardy, A.J. Lees, The expression of DJ-1 (*PARK7*) in normal human CNS and idiopathic Parkinson's disease, *Brain*. 127 (2004) 420–430.
- [5] J. Choi, M.C. Sullards, J.A. Olzmann, H.D. Rees, S.T. Weintraub, D.E. Bostwick, M. Gearing, A.I. Levey, L.-S. Chin, L. Li, Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases, *J. Biol. Chem.* 281 (2006) 10816–10824.
- [6] D. Antipova, R. Bandopadhyay, Expression of DJ-1 in neurodegenerative disorders, *Adv. Exp. Med. Biol.* 1037 (2017) 25–43.
- [7] C. Liu, X. Liu, J. Qi, O.P. Pant, C.-w. Lu, J. Hao, DJ-1 in ocular diseases: A review, *Int. J. Med. Sci.* 15 (2018) 430–435.
- [8] L. Zhang, M. Shimoji, B. Thomas, D.J. Moore, S.-W. Yu, N.I. Marupudi, R. Torp, I.A. Torgner, O.P. Ottersen, T.M. Dawson, V.L. Dawson, Mitochondrial localization of the Parkinson's disease related protein DJ-1: implications for pathogenesis, *Hum. Mol. Genet.* 14 (2005) 2063–2073.
- [9] N.K. Archibald, M.P. Clarke, U.P. Mosimann, D.J. Burn, The retina in Parkinson's disease, *Brain*. 132 (2009) 1128–1145.
- [10] R.S. Weil, A.E. Schrag, J.D. Warren, S.J. Crutch, A.J. Lees, H.R. Morris, Visual dysfunction in Parkinson's disease, *Brain*. 139 (2016) 2827–2843.
- [11] V.L. Bonilha, B.A. Bell, M.E. Rayborn, X. Yang, C. Kaul, G.H. Grossman, I.S. Samuels, J.G. Hollyfield, C. Xie, H. Cai, K.G. Shadrach, Loss of DJ-1 elicits retinal abnormalities, visual dysfunction, and increased oxidative stress in mice, *Exp. Eye Res.* 139 (2015) 22–36.
- [12] H. Büeler, Impaired mitochondrial dynamics and function in the pathogenesis of Parkinson's disease, *Exp. Neurol.* 218 (2009) 235–246.

- [13] X.-S. Zeng, W.-S. Geng, J.-J. Jia, L. Chen, P.-P. Zhang, Cellular and molecular basis of neurodegeneration in Parkinson disease, *Front. Aging Neurosci.* 10 (2018) 109.
- [14] J. Esteve-Rudd, L. Campello, M.-T. Herrero, N. Cuenca, J. Martín-Nieto, Expression in the mammalian retina of parkin and UCH-L1, two components of the ubiquitin-proteasome system, *Brain Res.* 1352 (2010) 70–82.
- [15] G.C. Martínez-Navarrete, J. Martín-Nieto, J. Esteve-Rudd, A. Angulo, N. Cuenca, α -Synuclein gene expression profile in the retina of vertebrates, *Mol. Vis.* 13 (2007) 949–961.
- [16] L. Campello, J. Esteve-Rudd, R. Bru-Martínez, M.T. Herrero, E. Fernández-Villalba, N. Cuenca, J. Martín-Nieto, Alterations in energy metabolism, neuroprotection and visual signal transduction in the retina of Parkinsonian, MPTP-treated monkeys., *PLoS One.* 8 (2013) e74439.
- [17] J. Esteve-Rudd, L. Fernández-Sánchez, P. Lax, E. De Juan, J. Martín-Nieto, N. Cuenca, Rotenone induces degeneration of photoreceptors and impairs the dopaminergic system in the rat retina, *Neurobiol. Dis.* 44 (2011) 102–115.
- [18] S. Oikawa, T. Yamada, T. Minohata, H. Kobayashi, A. Furukawa, S. Tada-Oikawa, Y. Hiraku, M. Murata, M. Kikuchi, T. Yamashima, Proteomic identification of carbonylated proteins in the monkey hippocampus after ischemia-reperfusion, *Free Radic. Biol. Med.* 46 (2009) 1472–1477.
- [19] A. Furukawa, Y. Kawamoto, Y. Chiba, S. Takei, S. Hasegawa-Ishii, N. Kawamura, K. Yoshikawa, M. Hosokawa, S. Oikawa, M. Kato, A. Shimada, Proteomic identification of hippocampal proteins vulnerable to oxidative stress in excitotoxin-induced acute neuronal injury, *Neurobiol. Dis.* 43 (2011) 706–714.
- [20] T.B. Sherer, R. Betarbet, J.H. Kim, J.T. Greenamyre, Selective microglial activation in the rat rotenone model of Parkinson's disease, *Neurosci. Lett.* 341 (2003) 87–90.
- [21] R. Betarbet, R.M. Canet-Aviles, T.B. Sherer, P.G. Mastroberardino, C. McLendon, J.H. Kim, S. Lund, H.M. Na, G. Taylor, N.F. Bence, R. Kopito, B.B. Seo, T. Yagi, A. Yagi, G. Klinefelter, M.R. Cookson, J.T. Greenamyre, Intersecting pathways to neurodegeneration in Parkinson's disease: Effects of the pesticide rotenone on DJ-1, α -synuclein, and the ubiquitin-proteasome system, *Neurobiol. Dis.* 22 (2006) 404–420.
- [22] O. Biehlmaier, M. Alam, W.J. Schmidt, A rat model of Parkinsonism shows depletion of dopamine in the retina, *Neurochem. Int.* 50 (2007) 189–195.
- [23] E.M. Normando, B.M. Davis, L. De Groef, S. Nizari, L.A. Turner, N. Ravindran, M. Pahlitzsch, J. Brenton, G. Malaguarnera, L. Guo, S. Somavarapu, M.F. Cordeiro, The retina as an early biomarker of neurodegeneration in a rotenone-induced model of Parkinson's disease: evidence for a neuroprotective effect of rosiglitazone in the eye and brain, *Acta Neuropathol. Commun.* 4 (2016) 86.
- [24] P. Lax, G. Esquivá, J. Esteve-Rudd, B.B. Ojalora, J.A. Madrid, N. Cuenca, Circadian dysfunction in a rotenone-induced parkinsonian rodent model, *Chronobiol. Int.* 29

- (2012) 147–156.
- [25] R.M. Canet-Avilés, M.A. Wilson, D.W. Miller, R. Ahmad, C. McLendon, S. Bandyopadhyay, M.J. Baptista, D. Ringe, G.A. Petsko, M.R. Cookson, The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 9103–9108.
- [26] Y. Saito, T. Miyasaka, H. Hatsuta, K. Takahashi-Niki, K. Hayashi, Y. Mita, O. Kusano-Arai, H. Iwanari, H. Ariga, T. Hamakubo, Y. Yoshida, E. Niki, S. Murayama, Y. Ihara, N. Noguchi, Immunostaining of oxidized DJ-1 in human and mouse brains, *J. Neuropathol. Exp. Neurol.* 73 (2014) 714–728.
- [27] M.R.R. Böhm, S. Mertsch, S. König, T. Spieker, S. Thanos, Macula-less rat and macula-bearing monkey retinas exhibit common lifelong proteomic changes, *Neurobiol. Aging.* 34 (2013) 2659–2675.
- [28] K.G. Shadrach, M.E. Rayborn, J.G. Hollyfield, V.L. Bonilha, DJ-1-dependent regulation of oxidative stress in the retinal pigment epithelium (RPE), *PLoS One.* 8 (2013) e67983.
- [29] X. Gu, N.J. Neric, J.S. Crabb, J.W. Crabb, S.K. Bhattacharya, M.E. Rayborn, J.G. Hollyfield, V.L. Bonilha, Age-related changes in the retinal pigment epithelium (RPE), *PLoS One.* 7 (2012) e38673.
- [30] N. Cuenca, M.-T. Herrero, A. Angulo, E. De Juan, G.C. Martínez-Navarrete, S. López, C. Barcia, J. Martín-Nieto, Morphological impairments in retinal neurons of the scotopic visual pathway in a monkey model of Parkinson's disease, *J. Comp. Neurol.* 493 (2005) 261–273.
- [31] M. Vila, S. Vukosavic, V. Jackson-Lewis, M. Neystat, M. Jakowec, S. Przedborski, α -Synuclein up-regulation in substantia nigra dopaminergic neurons following administration of the parkinsonian toxin MPTP, *J. Neurochem.* 74 (2000) 721–729.
- [32] O. Chiba-Falek, G.J. Lopez, R.L. Nussbaum, Levels of alpha-synuclein mRNA in sporadic Parkinson disease patients, *Mov. Disord.* 21 (2006) 1703–1708.
- [33] C.H. Carriere, N.H. Kang, L.P. Niles, Bilateral upregulation of α -synuclein expression in the mouse substantia nigra by intracranial rotenone treatment, *Exp. Toxicol. Pathol.* 69 (2017) 109–114.
- [34] P. Liu, L. Sun, X.L. Zhao, P. Zhang, X.M. Zhao, J. Zhang, PAR2-mediated epigenetic upregulation of α -synuclein contributes to the pathogenesis of Parkinsons disease, *Brain Res.* 1565 (2014) 82–89.
- [35] K.C. Wu, H.H. Liou, C.Y. Lee, C.J. Lin, Down-regulation of natural resistance-associated macrophage protein-1 (Nramp1) is associated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)/1-methyl-4-phenylpyridinium (MPP⁺)-induced α -synuclein accumulation and neurotoxicity, *Neuropathol. Appl. Neurobiol.* 45 (2019) 157–173.
- [36] C. Cook, L. Petrucelli, A critical evaluation of the ubiquitin-proteasome system in

- Parkinson's disease, *Biochim. Biophys. Acta.* 1792 (2009) 664–675.
- [37] B.I. Giasson, J.E. Duda, I. V Murray, Q. Chen, J.M. Souza, H.I. Hurtig, H. Ischiropoulos, J.Q. Trojanowski, V.M. Lee, Oxidative damage linked to neurodegeneration by selective α -synuclein nitration in synucleinopathy lesions, *Science.* 290 (2000) 985–989.
- [38] S. Krishnan, E.Y. Chi, S.J. Wood, B.S. Kendrick, C. Li, W. Garzon-Rodriguez, J. Wypych, T.W. Randolph, L.O. Narhi, A.L. Biere, M. Citron, J.F. Carpenter, Oxidative dimer formation is the critical rate-limiting step for parkinson's disease α -synuclein fibrillogenesis, *Biochemistry.* 42 (2003) 829–837.
- [39] L. Campello, J. Esteve-Rudd, N. Cuenca, J. Martín-Nieto, The ubiquitin-proteasome system in retinal health and disease, *Mol. Neurobiol.* 47 (2013) 790–810.
- [40] Z. Xu, D. Cawthon, K.A. McCastlain, W. Slikker Jr., S.F. Ali, Selective alterations of gene expression in mice induced by MPTP, *Synapse.* 55 (2005) 45–51.
- [41] E.-K. Tan, V.R. Chandran, S. Fook-Chong, H. Shen, K. Yew, M.-L. Teoh, Y. Yuen, Y. Zhao, Alpha-synuclein mRNA expression in sporadic Parkinson's disease, *Mov. Disord.* 20 (2005) 620–623.
- [42] M.S. Angeline, P. Chaterjee, K. Anand, R.K. Ambasta, P. Kumar, Rotenone-induced parkinsonism elicits behavioral impairments and differential expression of parkin, heat shock proteins and caspases in the rat, *Neuroscience.* 220 (2012) 291-301.
- [43] M. Barrachina, E. Castaño, E. Dalfó, T. Maes, C. Buesa, I. Ferrer, Reduced ubiquitin C-terminal hydrolase-1 expression levels in dementia with Lewy bodies, *Neurobiol. Dis.* 22 (2006) 265–273.
- [44] C.J. Werner, R. Heyny-von Haussen, G. Mall, S. Wolf, Proteome analysis of human substantia nigra in Parkinson's disease, *Proteome Sci.* 6 (2008) 8.
- [45] M. Inden, Y. Kitamura, H. Takeuchi, T. Yanagida, K. Takata, Y. Kobayashi, T. Taniguchi, K. Yoshimoto, M. Kaneko, Y. Okuma, T. Taira, H. Ariga, S. Shimohama, Neurodegeneration of mouse nigrostriatal dopaminergic system induced by repeated oral administration of rotenone is prevented by 4-phenylbutyrate, a chemical chaperone, *J. Neurochem.* 101 (2007) 1491–1504.
- [46] N. Lev, D. Ickowicz, E. Melamed, D. Offen, Oxidative insults induce DJ-1 upregulation and redistribution: implications for neuroprotection, *Neurotoxicology.* 29 (2008) 397–405.
- [47] C.M. Testa, T.B. Sherer, J.T. Greenamyre, Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures, *Brain Res. Mol. Brain Res.* 134 (2005) 109–118.
- [48] S. Shendelman, A. Jonason, C. Martinat, T. Leete, A. Abeliovich, DJ-1 Is a redox-dependent molecular chaperone that inhibits α -synuclein aggregate formation, *PLoS Biol.* 2 (2004) e362.
- [49] L. Zondler, L. Miller-Fleming, M. Repici, S. Gonçalves, S. Tenreiro, R. Rosado-Ramos,

- C. Betzer, K.R. Straatman, P.H. Jensen, F. Giorgini, T.F. Outeiro, DJ-1 interactions with α -synuclein attenuate aggregation and cellular toxicity in models of Parkinson's disease, *Cell Death Dis.* 5 (2014) e1350.
- [50] K.J. Thomas, M.K. McCoy, J. Blackinton, A. Beilina, M. Van der Brug, A. Sandebring, D. Miller, D. Maric, A. Cedazo-Minguez, M.R. Cookson, DJ-1 acts in parallel to the PINK1/parkin pathway to control mitochondrial function and autophagy, *Hum. Mol. Genet.* 20 (2011) 40–50.
- [51] W. Zhou, M. Zhu, M.A. Wilson, G.A. Petsko, A.L. Fink, The oxidation state of DJ-1 regulates its chaperone activity toward α -synuclein, *J. Mol. Biol.* 356 (2006) 1036–1048.
- [52] V.L. Bonilha, B.A. Bell, M.E. Rayborn, I.S. Samuels, A. King, J.G. Hollyfield, C. Xie, H. Cai, Absence of DJ-1 causes age-related retinal abnormalities in association with increased oxidative stress, *Free Radic. Biol. Med.* 104 (2017) 226–237.

FIGURE LEGENDS

Fig. 1. Expression of the DJ-1 gene in the retina of mammals. RT-PCR analysis of *DJ1* mRNA (A) and Western blotting analysis of DJ-1 protein (B) expression in the retinal pigment epithelium (RPE) and neural retina (NR) fractions from the indicated mammalian species. Molecular sizes of bands obtained are indicated on the right of each panel.

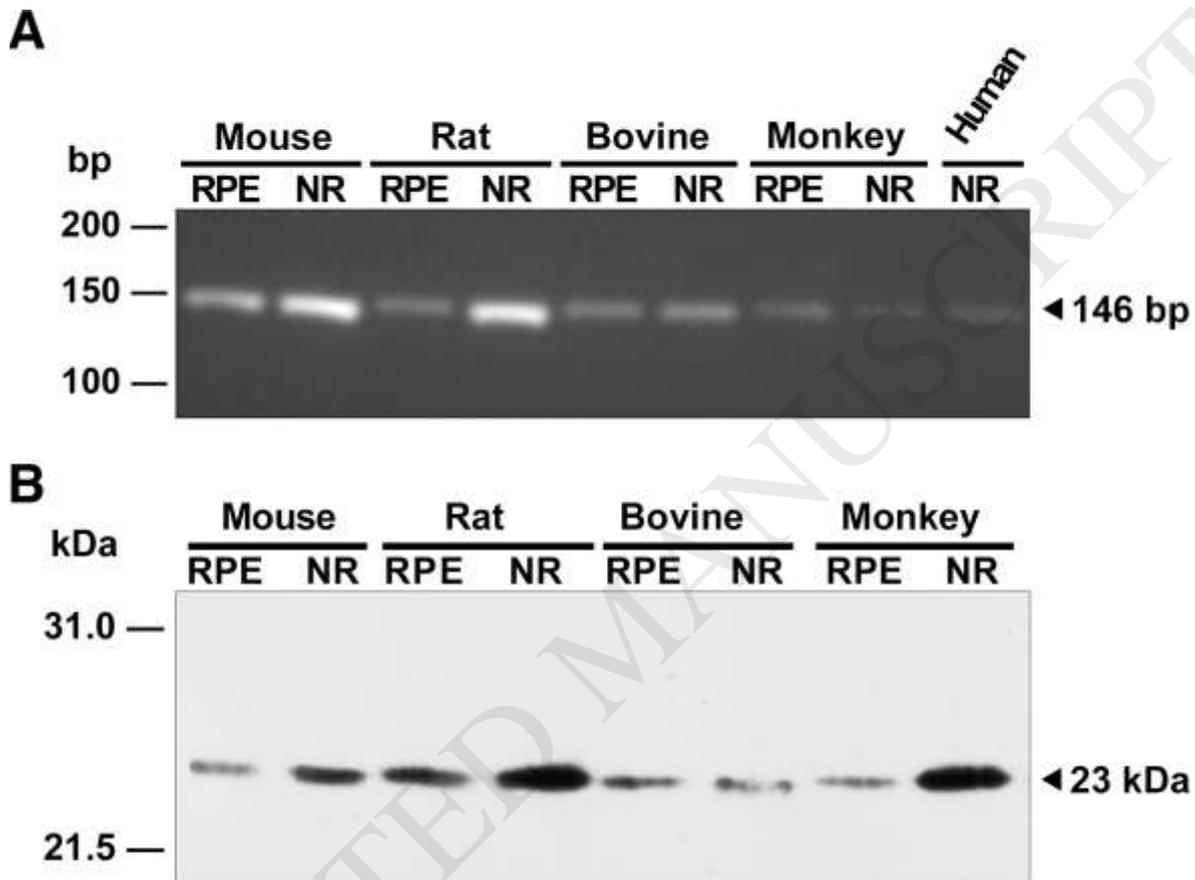


Fig. 2. Expression levels of PD-associated genes and their protein products in the neural retina of control and rotenone-treated rats. (A,B) Western blotting analysis of α -synuclein protein levels. Signal intensities obtained in the blots for α -synuclein and β -actin are shown in panel A, and α -synuclein levels normalized to β -actin levels from control, untreated rats (Ctrl), taken as 100%, are shown in B in comparison to normalized levels of rotenone-treated rats (Rot). (C) qRT-PCR analysis of *Snca* mRNA expression levels, normalized to *Gapdh* mRNA levels, in the retina of control and rotenone-treated rats. (D,E) Western blotting analysis of parkin protein levels. Signals obtained in the blots for parkin and β -actin are shown in D, and normalized parkin levels from control and treated rats are shown in E. The black arrowhead in D indicates the canonical parkin isoform,

of 52 kDa, and the white arrowhead the previously-reported 48 kDa alternative parkin isoform [14]. **(F,G)** Western blotting analysis of DJ-1 protein levels. Signals obtained in the blots for DJ-1 and β -actin are shown in **F**, and normalized DJ-1 levels from control and treated rats are shown in **G**. **(H)** qRT-PCR analysis of *Dj1* mRNA levels, normalized to *Gapdh* gene mRNA levels, in the retina of control and treated rats. **(I,J)** Western blotting analysis of UCH-L1 protein levels. Signals obtained in the blots for UCH-L1 and β -actin are shown in **I**, and normalized UCH-L1 levels from control and treated rats are shown in **J**. All histograms (panels **B,C,E,G,H,J**) represent the average \pm SEM values obtained from 3-5 individuals. Differences between expression levels were evaluated by the Student's *t*-test (*, significant, $p < 0.05$; n.s., not significant, $p > 0.05$).

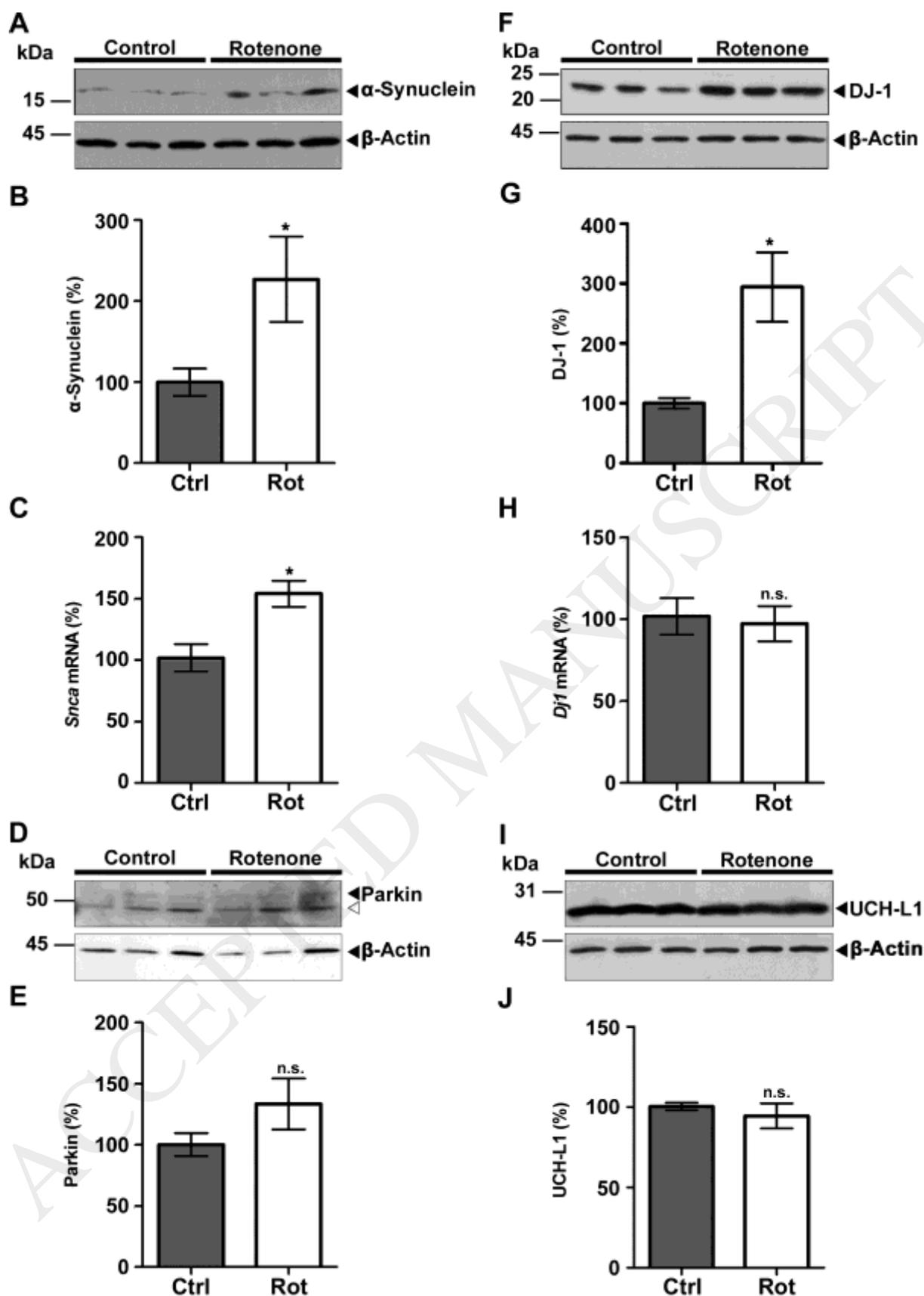
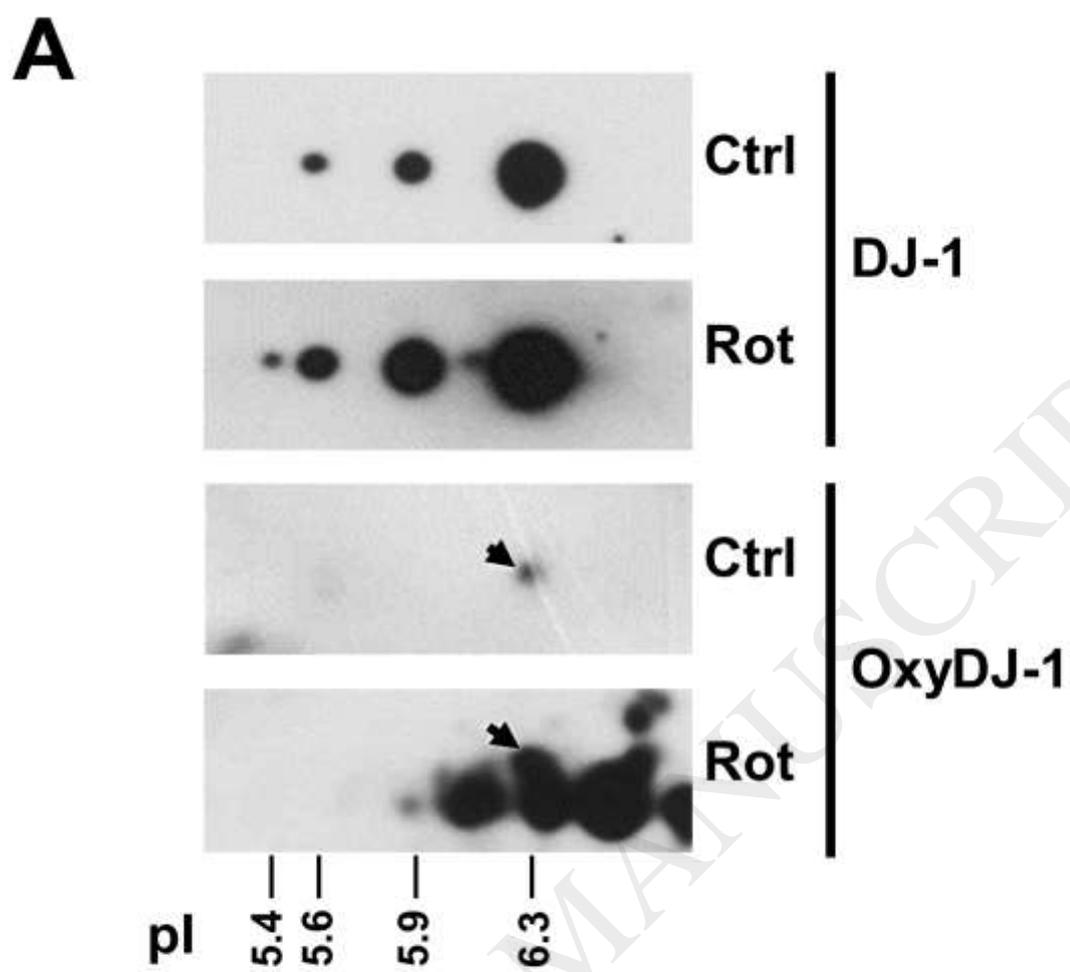


Fig. 3. Overexpression and oxidative modification of DJ-1 in the rat neural retina upon rotenone treatment. (A) Western blotting analysis of DJ-1 protein levels in control (Ctrl) and rotenone-treated (Rot) rat retinal extracts subjected to 2D gel electrophoresis. The

1st and 2nd panels show the results obtained after probing extracts from control and rotenone-treated rats, respectively, with antibodies to DJ-1. The 3rd and 4th panels show the results obtained after analysis with anti-DNP antibodies of oxidized proteins present in control and rotenone-treated rat extracts, respectively, after derivatization of carbonyl groups with DNPH. The arrowhead indicates a DJ-1 protein spot with an apparent Mr of 23 kDa and a pI of 6.3 which exhibited an increased oxidation (OxyDJ-1) in rotenone-treated rat retinas with respect to control animals. **(B)** The relative levels of this pI 6.3 DJ-1 isoform were measured by densitometric quantitation of spot intensity in control (Ctrl) and rotenone-treated (Rot) rats (n = 4 each) using the NIH ImageJ software, taking control levels as 100%. Bars represent the average \pm SEM values. Differences were evaluated by the Student's *t*-test (*, significant, $p < 0.05$).



B

