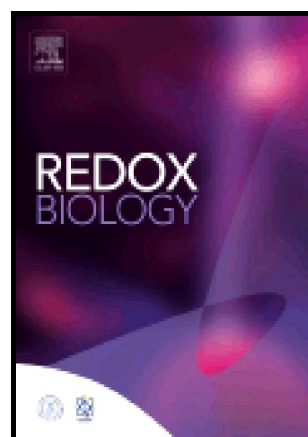


## Author's Accepted Manuscript

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IGF-II promotes neuroprotection and neuroplasticity recovery in a long-lasting model of oxidative damage induced by glucocorticoids

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## Abstract

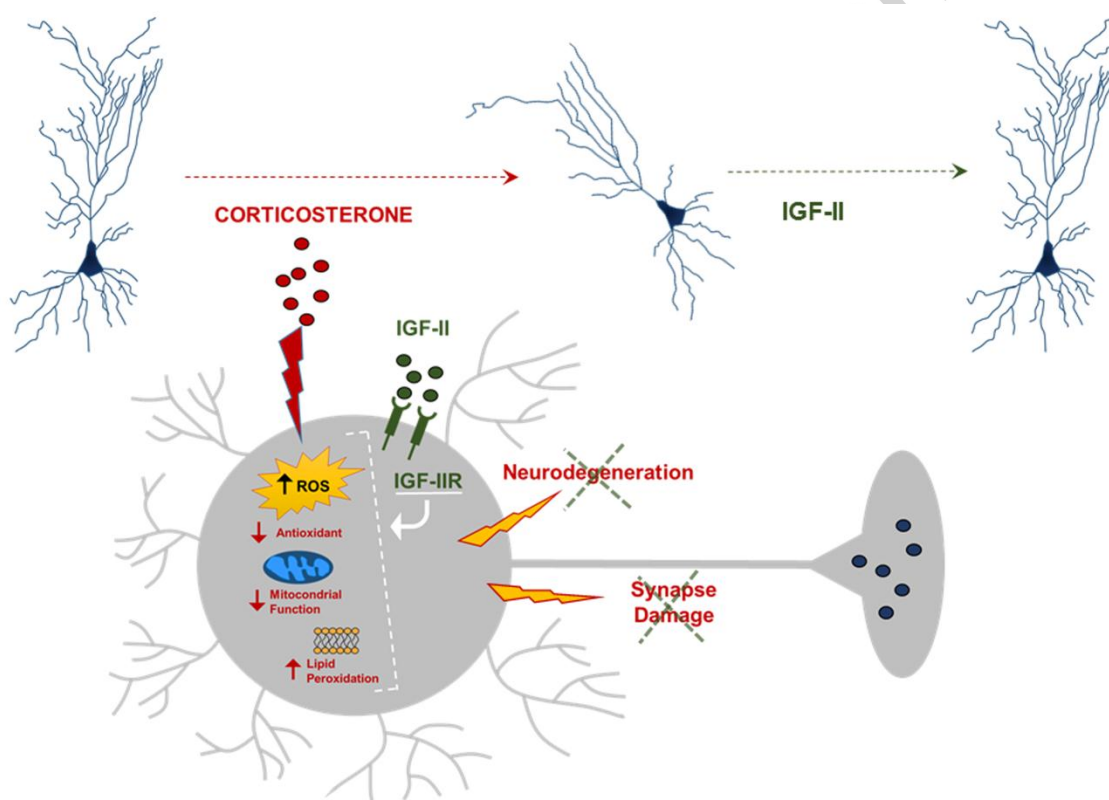
Insulin-like growth factor-II (IGF-II) is a naturally occurring hormone that exerts neurotrophic and neuroprotective properties in a wide range of neurodegenerative diseases and ageing. Accumulating evidence suggests that the effects of IGF-II in the brain may be explained by its binding to the specific transmembrane receptor, IGFII/M6P receptor (IGF-IIR). However, relatively little is known regarding the role of IGF-II through IGF-IIR in neuroprotection. Here, using adult cortical neuronal cultures, we investigated whether IGF-II exhibits long-term antioxidant effects and neuroprotection at the synaptic level after oxidative damage induced by high and transient levels of corticosterone (CORT). Furthermore, the involvement of the IGF-IIR was also studied to elucidate its role in the neuroprotective actions of IGF-II. We found that neurons treated with IGF-II after CORT incubation showed reduced oxidative stress damage and recovered antioxidant status (normalized total antioxidant status,

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<sup>1</sup> These authors have equally contributed.

lipid hydroperoxides and NAD(P) H:quinone oxidoreductase activity). Similar results were obtained when mitochondria function was analysed (cytochrome c oxidase activity, mitochondrial membrane potential and subcellular mitochondrial distribution). Furthermore, neuronal impairment and degeneration were also assessed (synaptophysin and PSD-95 expression, presynaptic function and FluoroJade B® stain). IGF-II was also able to recover the long-lasting neuronal cell damage. Finally, the effects of IGF-II were not blocked by an IGF-IR antagonist, suggesting the involvement of IGF-IIR. Altogether these results suggest that, in our model, IGF-II through IGF-IIR is able to revert the oxidative damage induced by CORT. In accordance with the neuroprotective role of the IGF-II/IGF-IIR reported in our study, pharmacotherapy approaches targeting this pathway may be useful for the treatment of diseases associated with cognitive deficits (i.e., neurodegenerative disorders, depression, etc.).

Graphical abstract



## Keywords

Insulin-like growth factor-II; Insulin-like growth factor-II receptor; Oxidative stress; Neuroprotection; Mitochondria; Synapsis.

## 1. INTRODUCTION

IGF-II is a pleiotropic hormone that can act as an autocrine, paracrine and endocrine factor. Its functions are regulated by binding to IGF receptors and binding proteins 1-6 [1,2]. Recently, it has been proposed that the biological effects of IGF-II occur through specific IGFII/M6P receptor interactions (IGF-IIR) [3]. Notwithstanding, IGF-II actions mediated by IGF-IIR have not yet been clearly established. The role of IGF-IIR in enzyme lysosomal trafficking from the trans-Golgi network to endosome for activation and/or clearance of growth factors and degradation of IGF-II mediated by endocytosis are well known [4]. In the central nervous system (CNS), the biological effects of IGF-II interacting with its specific receptor are relevant not only for metabolism, growth and development (mainly survival and differentiation) but also for neurotransmitter release, memory consolidation and neuroprotection under several pathological conditions [5–10].

Recently, it has been suggested that IGF-II could be an interesting neuroplastic factor and a good candidate for the treatment of diseases in which cognitive impairment is present. This proposal is consistent with a recent finding that IGF-II/IGF-IIR signalling promotes synapse formation and spine maturation in the mouse brain [11]. Moreover, administration of IGF-II also rescued spine formation and excitatory synaptic function in the hippocampus of a mouse model of Alzheimer's disease [12] and can modulate memory processes, likely acting at different levels on synapses [9,13–17].

In a previous work, we demonstrated the antioxidant and neuroprotective effect of IGF-II via IGF-IIR on oxidative damage and mitochondrial function in cortical neurons treated with transient high levels of corticosterone (CORT)[18]. CORT increases ROS production and impairs mitochondrial functions, leading to lipid, protein and nucleic acid damage [19]. Oxidative stress induced by high levels of glucocorticoids reduces neuronal survival and alters numerous aspects of neuronal functions, playing an important role in maintaining the balance between adaptation and excitotoxicity to stress [20]. In fact, high levels of corticoids induce marked damage in mitochondria, enhance extracellular levels of glutamate and increase excitotoxicity [21,22]. As a consequence of oxidative stress, both the function and transport of mitochondria to synaptic regions are altered, impairing synaptic function [23–25], which results in neurodegeneration [26]. Furthermore, a close interplay between synapses and mitochondria is clear. Mitochondria are located in synaptic terminals and tethered to sites where neurotransmitter release occurs [27]. In functional synapses, mitochondria

supply energy and regulate calcium levels, modulating neuronal polarity [28], synaptic neurotransmission [29], receptor signalling [30], and synaptic plasticity [24,31]. Toxic levels of corticoids are also related with impaired synaptic functions and cognition [32], decreased neurogenesis and spine density causing dendritic atrophy [33]. In this regard, it has also been suggested that the deleterious effect of CORT on learning and memory processes is mediated by the interaction of CORT with specific receptors located mainly at the prefrontal cortex [34]. Furthermore, damage produced by oxidative stress and/or high levels of corticoids in specific cortical synaptic proteins results in cognitive impairment as seen in common neuropsychiatric disorders such as depression, Alzheimer's disease and Parkinson's disease [34–36]. In addition, synaptic dysfunctions have also been shown in other psychiatric conditions, including autism, schizophrenia and bipolar disorder [25,37,38].

Although we have previously described the capacity of IGF-II through its IGF-IIR to prevent the neuronal oxidative damage induced by CORT [18], its role in neuroprotection at the synaptic level and recovery from this oxidative damage, has not yet been studied. Taking all this into account, in the present study, we hypothesized that IGF-II, primarily via the IGF-IIR, is able to recover cortical neurons from the synaptic damage mediated by ROS in a cellular model of neurotoxicity induced by high and transient levels of CORT. Our results show that IGF-II provides remarkable neuroprotection at the synaptic level in cortical primary neurons treated with corticoids. Furthermore, this protective effect is at least partially explained by the stimulation of the IGF-IIR. In our opinion, the outcomes of this work may contribute to the development of new therapeutic strategies in which IGF-II may be the key for the treatment of cognitive impairment linked to neurodegenerative and neuropsychiatric disorders [16,39].

## 2. MATERIAL AND METHODS

### 2.1. Reagents

All reagents used for cell culture were provided by Life Technologies (Madrid, Spain) and SIGMA-ALDRICH (Madrid, Spain). CORT, phosphatase and protease inhibitors, COX assay kit, primary antibody against  $\beta$ -tubulin, secondary antibodies, Advasep-7 (scavenger of FM dyes), CNQX (AMPA receptor antagonist) and AP5 (NMDA receptor antagonist) were obtained by SIGMA-ALDRICH. Primary antibodies against synaptophysin, PSD-95 and FJ (Fluoro-Jade B®) dye were provided by Merk Millipore (Darmstadt, Germany) and Santa Cruz Biotechnology Inc. (Heidelberg, Germany),

respectively. IGF-II was provided by Lilly Laboratories (Madrid, Spain). IGF-IR antagonist (JB1) was purchased from Bachem (Bubendorf, Switzerland). A Total Antioxidant Status (TAS) kit was purchased from Randox Laboratories Ltd. (Antrim, UK). JC-1 (5,5,6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbo-cyanine iodide), FM®1-43 (N-(3-Triethylammoniumpropyl)-4-(4-(Dibutylamino) Styryl) Pyridinium Dibromide) dyes and secondary antibody Alexa Fluor® 488 were purchased from Molecular Probes, Thermo Fisher Scientific (Madrid, Spain). All other chemicals were obtained from SIGMA-ALDRICH unless stated otherwise.

## 2.2. Cortical neuronal cultures

Cultures of cortical neurons from adult rats were prepared according to a previously published procedure with minor modifications [40]. The cortex was removed, and slices of approximately 0.5 mm were transferred to tubes containing Hibernate®-A/B27® supplement and papain at the appropriate concentration and incubated for 30 min in a 30 °C water bath with gentle shaking. Then, the slices were triturated 10×3 times. The cells obtained from the sections were loaded in OptiPrep gradient medium and centrifuged at 800 *g* for 15 min. The fractions containing neurons were collected, suspended in Hibernate®-A/B27 and centrifuged twice at 200 *g* for 2 min. The pellets were then resuspended in growth culture medium (Neurobasal®-A/B27 containing glutamine, FGF2 and penicillin/streptomycin). For cell culture, 6- and 24-well plates were pre-coated with 100 µg/mL poly-D-lysine. For immunohistochemical studies, glass coverslips (12 mm diameter) were pretreated with polyethylenimine (1/500 v/v in ddH<sub>2</sub>O) followed by 2.5% FBS. Approximately 500,000 (6-well plates) or 125,000 (24-well plates) cells were seeded. The neurons were then incubated at 37 °C in 5% CO<sub>2</sub>, and after 1 h, the wells were rinsed twice with Neurobasal®-A and filled with growth culture medium. The media were replaced with fresh media (half volume) every 4 days. The experiments were performed after 12 days of growth. In the present study, we used serum-free media, and therefore, the factors associated with the IGF system, such as IGFBPs [41,42] and other proteases such as Htra1[43], that could influence the interaction of IGF with its receptors or modulate the bioavailability of IGF were not present. The absence of these factors in the cultures allowed us to more accurately interpret the results.

All experimental procedures were performed in accordance with European animal research laws (European Communities Council Directive 2010/63/EU, 90/219/UE, 90/219/CEE, Regulation (EC) No. 1946/2003) and the Spanish National Guidelines for Animal Experimentation (Real Decreto 1201/2005 and Ley 32/2007). All animal

procedures were approved by the Institutional Animal Care and Use Committee of Malaga University (CEUMA 08-7-15-274).

### *2.3. Cellular treatment*

To avoid the antioxidant properties of B27, this supplement was removed from the culture media, and the cells were maintained under this condition for 24 hours before the experiments. All the incubations were at 37 °C unless stated. The cell cultures were exposed to:

- 10  $\mu$ M CORT for 2 hours (CORT-2 group).
- 10  $\mu$ M CORT for 2 hours and after that, cells were washed and maintained for 22 additional hours in Neurobasal®-A/-B27 culture medium, alone (WCORT-24) or containing 100 ng/mL IGF-II (WCORT-24+IGF-II) or 100 ng/mL IGF-II in the presence of 20 ng/ $\mu$ L of the IGF-IR antagonist JB1 (WCORT-24+IGF-II+JB1);
- 10  $\mu$ M CORT during 2 hours, washed and treated during 2 more hours with 100 ng/mL IGF-II (CORT-2+IGF-II-2) and finally we replicate this last group but maintaining after the incubation with IGF-II and wash, the cells for 20 more hours with Neurobasal®-A/-B27 culture medium (WCORT-24+IGF-II-2).
- Neurobasal®-A/-B27 culture medium for 2 hours, then cells were washed and treated with 100 ng/mL IGF-II, during 22 hours in the same conditions than above (CO+IGF-II).

All the measurements were performed immediately after treatments.

### *2.4. Preparation of homogenised cells from cortical neuronal cultures*

The cells were suspended in buffer containing 10 mM HEPES, pH 7.4, 10 mM KCl, a protease inhibitor cocktail and phosphatase inhibitors, incubated at 0 °C for 20 min and homogenised in the presence of 0.01% digitonin. The protein concentrations were determined via quantification of UV 280 nm absorbance using a Nanodrop® system (Thermo Scientific NanoDrop Products, USA). Cytochrome c oxidase (COX), NAD(P) H:quinone oxidoreductase [NAD(P)H-(quinone acceptor) oxidoreductase] (EC 1.6.99.2; NQO1) activity, lipid hydroperoxides (LOOH) levels and Total Antioxidant Status (TAS) (see below) were determined in the homogenates. Western blot analyses were performed using the same homogenates without incubation in digitonin.

### *2.5. Markers of oxidative stress*

LOOHs were measured using the FOX2 method adapted to a Cobas Mira Autoanalyzer (Roche, Basel, Switzerland) [44]. The LOOHs level was calculated relative to a hydrogen peroxide standard curve and was expressed as nmol/mg of protein. The TAS, i.e., the total enzymatic and non-enzymatic antioxidant capacity, was evaluated in cells using a commercial TAS kit adapted to a Cobas Mira Autoanalyzer that measures the formation of the radical cation ABTS<sup>•+</sup> at 600 nm using ABTS<sup>•+</sup> in the presence of a peroxidase (metmyoglobin) and H<sub>2</sub>O<sub>2</sub> [18,45]. The final values were expressed as μmol/mg of protein.

NQO1 activity (EC 1.6.99.2) was measured as described elsewhere[46] by following the decrease in NADH absorbance at 340 nm adapted to a Cobas Mira Autoanalyzer. The reaction mixture at a final volume of 200 μL contained 25 mM Tris·HCl (pH 7.5), 0.01% Tween 20, 0.7 mg/mL BSA (pH 7.4), 40 μM menadione, 5 μM FAD, 200 μM NADH, and cell extract. Measurements were made at 25 s intervals over a time period of 10 min. To determine NQO1 activity in cell extracts, cells were washed with PBS and extracted by sonication in lysis buffer, and the decrease in NADH absorbance was measured in the absence and presence of 10 μM dicoumarol to measure specific NQO1 activity.

#### *2.6. Measurement of mitochondrial function markers*

As mitochondrial function markers, we chose the mitochondrial membrane potential (MMP) and enzyme cytochrome c oxidase (EC 1.9.3.1; COX). MMP was evaluated using the lipophilic cationic probe JC-1 according to a previously described procedure [47]. JC-1 is a lipophilic carbocyanine that exists in a monomeric form and accumulates in mitochondria. In the presence of a high MMP, JC-1 reversibly forms aggregates that, after excitation at 488 nm, fluoresce orange/red (590 nm). Collapse of the MMP provokes a decrease in the number of JC-1 aggregates (see cells incubated in valinomycin) and a subsequent increase in monomers that fluoresce green (525 nm). This phenomenon is detected as a decrease in orange/red fluorescence and/or an increase in green fluorescence. The cells were incubated in 1 μg/mL JC-1 in B27-free medium for 20 min at 37 °C. Then, the cells were rinsed twice with B27-free medium and observed under a confocal microscope LEICA SP5 II (Wetzlar, Germany) with excitation at 488 nm and emission at ~530 nm for green JC1 monomers and ~590 nm for JC1 aggregates. The images to be evaluated are separated into their red, green and blue components. To eliminate background noise, a minimum mark threshold was set, the red and green components were segmented according to this threshold, and binary images containing only the marked pixels corresponding to soma and



dendrites/axon were obtained. To evaluate the signal in the soma, an erosion and dilation operation was carried out that eliminates the dendrites/axon. A new set of binary images was thus obtained. All binary images were used as a mask to determine the total intensity of the pixels measured on the original images. In addition, the colocalization of both signals, red and green, was measured. All the images were processed using the ImageJ plugin colour 3D viewer (Fiji Is Just ImageJ) to analyse hue, saturation and brightness (HSB) and these values were used to calculate the red/green ratio [48].

COX activity (EC 1.9.3.1) in cell homogenates was assessed using a COX assay kit adapted to a Cobas Mira Autoanalyzer [49]. One unit was defined as the oxidation of 1.0  $\mu\text{mol}$  of ferrocytochrome c per minute at pH 7.0 and 37 °C.

### *2.7. Electrophoresis and Western blot*

The samples were resuspended in (5X) polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiled at 100 °C for 3 min using a thermo-block. The samples were then loaded (15  $\mu\text{g}$  of protein/well) on a 12% polyacrylamide gel and subjected to a constant current of 130 V. The transfer was performed using a semi-dry transfer device (Multiphor II®, GE Healthcare Life Sciences, Madrid, Spain) on graphite plates to nitrocellulose membranes with a pore size of 0.45  $\mu\text{m}$  (current intensity: 0.8  $\text{mA}/\text{cm}^2$  for 45 min). After blocking, the membranes were incubated in various primary antibodies (produced in rabbit) at different dilutions (anti-synaptophysin (1:500) and anti- $\beta$ -tubulin neuronal (1:750)) for 12 hours at 4 °C, followed by incubation for 1 hour with the anti-rabbit IgG alkaline phosphatase-conjugated secondary antibody at a 1:5000 dilution. The final colour reaction was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP). The Western blots were digitised using a flatbed scanner (HP Scanjet 5500c, Hewlett-Packard, Madrid, Spain) and analysed using ImageJ software (US National Institute of Health; <http://imagej.nih.gov/ij/>).

### *2.8. Immunocytochemical staining of cortical neurons*

Fixation was accomplished by treating the plate with methanol previously chilled to -20 °C and incubating at -20 °C for 20 min. The wells were washed with PBS, and the coverslips were removed and incubated in a primary antibody solution containing the following: goat anti-PSD-95 polyclonal antibody (1:250) in PBS, 3% BSA and 0.02% sodium azide at 4 °C overnight. Then, the coverslips were incubated with the secondary antibody Alexa Fluor® 488-conjugated donkey anti-goat IgG (1:200) in PBS

for 1 hour at room temperature in the dark. After fixation as described above, the coverslips were mounted using Fluoromount®. Images were acquired using a LEICA SP5 II Microscope. The image-analysis software used was LAS AF Lite. PSD-95 immunoreactivity intensity (IR) is indicative of the accumulation of this protein at the post-synaptic terminal [50]. The PSD-95 IR semi-quantification is expressed as the average of PSD-95 IR intensity of individual puncta in 25 positive cells per condition. Four independent experiments were performed. The application settings were adjusted at the beginning of the analysis and kept the same for all images in the experiment.

### *2.9. Assessment of presynaptic terminal function*

To assay the functional nerve terminals, we used the changes in FM®1-43 fluorescence intensity based on imaging methods. To measure the endocytosis process, neurons were bathed in an extracellular solution containing the FM®1-43 dye, enabling it to be taken up into synaptic vesicles (SVs). To assay the subsequent exocytosis or destaining process, the dye was washed out by applying a dye-free extracellular solution leading to loss of the FM®1-43 dye from the inside to the extracellular space. Staining and destaining were triggered by extensively stimulating the neurons. In brief, neurons were loaded with 10  $\mu$ M FM®1-43 in Tyrode's solution by field stimulation (5 min, 10 Hz; 200  $\mu$ s, 0.4 mA) [51,52]. The destaining was triggered by the same electrical field stimulation maintained during 15 min immediately after the wash out of the dye. To reduce the background, stimulation of the exocytosis process was performed in the presence of 1 mM Advasep-7, a modified cyclodextrin that serves as a scavenger of FM dyes from the plasma membrane (Figure S1). To avoid the loss of FM dye through synaptic activity, we added an antagonist of AMPA (10  $\mu$ M CNQX) and NMDA receptors (50  $\mu$ M AP5).

Images were analysed by calculating the changes in FM intensity at the start and end of the series ( $\Delta$ FM). The FM® 1–43-associated fluorescence was calculated by subtracting the fluorescence intensity in the unloaded frame (representing nonspecific staining) from the averaged intensity of the first two loaded frames. We assigned regions of interest (ROI) to FM puncta that putatively correspond to nerve terminals, and measured changes in ROI intensity over time. This represents the cumulative amount of evoked synaptic activity. Images were acquired using a LEICA SP5 microscope at 520 nm and 490 nm (excitation and emission filters, respectively). We used LAS AF Lite software for the image-analysis as a stack of time-series images. To calculate the unloading rate, a fluorescence time course was generated by normalizing each ROI time course, dividing by the starting intensity and then dividing each ROI by bleaching at the corresponding time points throughout the experiment. The  $t_{1/2}$  decay of

intensity during unloading was calculated by fitting the points to a single exponential decay curve using GraphPad software [53].

#### 2.10. Assessment of neurodegeneration in cell cultures

Neurodegeneration of the cultured cortical neurons was measured using FJ dye according to a previously published procedure [18,54]. The cell culture plates were fixed using cold ethanol (-20 °C) for 30 min and washed three times with distilled water. The cells were treated with the dye (final concentration of 0.0004% FJ in 0.1% acetic acid), gently shaken for 30 min in the dark at room temperature and then washed twice with distilled water. Finally, the fluorescence intensity was determined using a FLUOstar Galaxy spectrophotometer (BMG, Lab Technologies, Ortenberg, Germany) at 485/530 nm.

#### 2.11. Statistical analysis

Statistical differences were determined using one-way ANOVA. Pairwise comparisons were performed using a *post hoc* Newman-Keuls multiple comparison test. Statistical significance was considered to be  $p < 0.05$ . For data in which the measured units were arbitrary, the respective values represent the percentage relative to the control value unless specified.

### 3. RESULTS

To evaluate the neuroprotective effect of IGF-II on neuronal synaptic function, we used an experimental model previously published based on the use of CORT to produce oxidative damage similar to that found in some neurodegenerative and neuropsychiatric disorders. In this model, we utilized adult rat cortical neuronal cultures incubated 2 hours under CORT-induced oxidative stress conditions (10  $\mu$ M). After oxidative damage, the cells were washed to remove CORT, and culture medium (Neurobasal/B27 free) was added to each well. Then, the cells were submitted to a further 22 hours incubation period in which IGF-II and JB1 were added to the samples before the measurements. This period of time was chosen in order to study whether IGF-II can protect cortical neurons against long-lasting synaptic impairment induced by CORT [55–57].

The IGF-II dose chosen in this experimental model was the same as that used in previous work in which it was shown to be neuroprotective and an antioxidant [18]. To

assess which IGF-R is involved in the protective IGF-II effect, the IGF-IR was blocked using the specific antagonist JB1, and several attempts were made to block the IGF-IIR with antibodies (anti-IGF-IIR, R&D Systems, USA), although no clear results were obtained likely because the antibody binding to its epitopes is affected by the long periods of incubation (22 hours) at 37 °C in a medium without sera or blocking proteins.

### *3.1. IGF-II ameliorates oxidative stress damage*

The TAS (i.e., the antioxidant power of cells for further anti-ROS activity) was significantly lower in cortical neurons incubated for 2 hours with 10 µM CORT (CORT-2) compared to control cells (CO). The decrease in intracellular TAS was evident even 22 hours after the elimination of CORT from the incubation medium of the cells (WCORT-24). However, when we added IGF-II to the culture medium after the removal of CORT, the levels of cellular TAS showed a significant increase in the IGF-II-treated group compared with WCORT-24; recovering the same activity as observed in control cells. In the same experiments, co-incubation of IGF-II with JB1 did not reverse the IGF-II effect seen (Figure 1a).

To confirm that, in this model, the cells undergo oxidative stress, we measured the cellular levels of lipid hydroperoxides (LOOHs). Cellular LOOHs significantly increase in cultured cells incubated with CORT for 2 hours compared to CO cells (CORT-2). Cellular LOOHs continue to significantly increase after 22 hours despite the elimination of CORT from the culture medium, as observed in the WCORT-24 cells compared to the CORT-2 cells and CO. Similar to the above finding, when we added IGF-II to the medium after CORT removal, we observed a significant decrease in LOOHs compared to WCORT-24, again reverting the effect of CORT and recovering values similar to those found in CO cells. Co-incubation of IGF-II in the presence of JB1 did not totally revert the IGF-II effect (Figure 1b).

When we analysed the downstream effector Nrf2 by measuring the activity of NQO1, the neuronal cells incubated with 10 µM CORT for 2 hours (CORT-2) showed a significant decrease in NQO1 activity compared with CO cells. NQO1 activity revealed a slight increase when CORT was removed from the medium in the WCORT-24 cells compared with CORT-2, but the activity remained significantly lower than in the CO cells. When we added IGF-II to the culture medium after incubation for 2 hours with CORT and its subsequent removal, the activity of NQO1 in these cells was very close to that in the CO cells. Again, co-incubation of IGF-II with JB1 did not reverse the IGF-II effect, and these cells maintained activities similar to those found in CO cells (Figure 1c).

Agreeing with previous results [18] incubation of control cells with IGF-II during 22 hours did not produce any change in the oxidative stress markers (TAS, LOOH, NQO1, Figure S2a).

In order to assess the duration of the effect of IGF-II on the oxidative damage produced by corticosterone, an additional experiment was performed in which we measured oxidative stress markers in 2 different situations: In the first one, cells were transiently incubated during 2 hours with IGF-II after corticoid damage; in the second one, after the above step, a set of cells were maintained for 20 additional hours without IGF-II in the incubation media (Figure S3a).

In the first situation, the results obtained showed that IGF-II (CORT-2+IGF-II-2) significantly decreases intracellular TAS values compared with CO, reaching values even lower than CORT-2 group. This trend to the decrease in TAS values was maintained and even amplified when IGF-II was removed from the media and the incubation maintained for 20 more hours (WCORT-24+IGF-II-2) ( $p < 0.05$  vs CORT-2+IGF-II-2), with values identical to those found when no IGF-II was added to the media (WCORT-24), as shown in the second situation. Conversely, this effect on TAS values gets inverted when IGF-II is present on the media during the 22 hour of total incubation time, recovering values similar to control cells (CO) (Figure S3b). The same behavior was seen in NQO1 (Figure S3c).

To assess the oxidative damage, LOOH was measured in the two above situations. The results obtained in these experiments show that IGF-II (CORT-2+IGF-II-2) produce a transient decrease in LOOH production compared with CORT-2 reaching values close to CO. This effect is not maintained on time, as seen when analyzing LOOH after 20 hours of incubation without IGF-II in the culture media ( $p < 0.05$  vs CORT-2+IGF-II-2); in the WCORT-24+IGF-II-2 group, LOOH values were higher than those found when IGF-II was maintained in the culture media ( $p < 0.05$  vs WCORT-24+IGF-II) and very close to the values obtained in CORT-2 and WCORT-24.

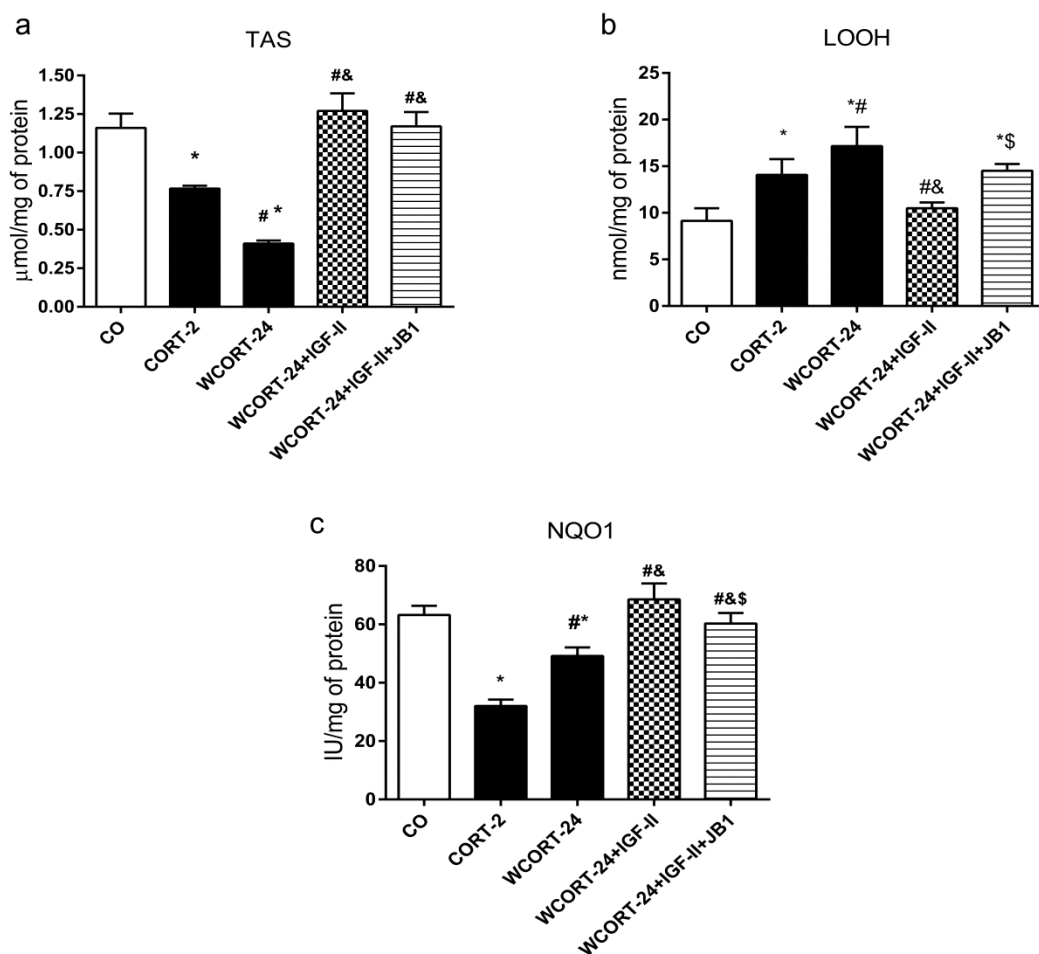


Figure 1. IGF-II restores REDOX imbalance produced by CORT treatment. Panel a shows the levels of the antioxidant TAS and panel b shows the level of the oxidative marker LOOH after different treatment conditions. Panel c shows the activity of the antioxidant NQO1. The data represent mean $\pm$ SEM from 3-4 independent experiments. (\*  $p < 0.05$  versus CO; &  $p < 0.05$  versus WCORT-24; #  $p < 0.05$  versus CORT-2; \$  $p < 0.05$  versus WCORT-24+IGF-II).

### 3.2. IGF-II changes neuronal mitochondrial function

Mitochondria are essential for metabolism, survival and neuronal function including the mobilization of synaptic vesicles for exocytosis and recycling and are structurally distributed in different functional domains into the cell body, axons and dendrites.

The use of the mitochondrial sensitive probe JC-1 allowed us to study the MMP in whole cells and the subcellular heterogeneity along neurons. Confocal microscopy images were processed in the HSB (hue, saturation, brightness) colour space and extended to three-dimensional images in order to perform a more objective analysis. In these experiments, we observed a switch from red to green fluorescence along with an increase in green brightness in the treated WCORT-24 cells versus CO. When CORT

was removed from the medium and IGF-II was added, we observed a new switch to orange as a result of an increase in red fluorescence and a decrease in green fluorescence. No further changes were seen when the IGF-IR was blocked by adding JB1 to the incubation medium. These changes reflect the polarization state of the mitochondrial membrane. In CO cells, where the membrane is polarized, the distribution and brightness of red is higher than in CORT-treated cells (WCORT-24), in which the mitochondrial membranes are partially depolarized as seen by the increase in green brightness. When CORT was removed from the medium and IGF-II was added, we partially avoided the depolarization promoted by CORT, recovering values close to those found in CO cells. The blockage of the IGF-IR with JB1 does not modify these changes in mitochondrial membrane potential (Figure 2a-d).

When a more detailed analysis using confocal microscopy was performed, we observed in WCORT-24 cells an increase in the number of less polarized mitochondria near the soma, as shown by a decline in the red/green ratio fluorescence ( $p < 0.05$  vs CO) (Figure 2e). When IGF-II was added to the culture medium, the red/green ratio fluorescence increased to control levels. The addition of JB1 did not induce statistically significant changes on the effect of IGF-II.

In the non soma compartment, we observed a similar behaviour than above, with a decrease in orange fluorescence (ratio red/green) in cells treated with WCORT-24 *versus* CO (Figure 2f). When IGF-II was added to the culture media, a statistically significant increase in orange fluorescence was seen, and this increase was higher when the IGF-IR was blocked with JB1, with intensities close to those seen in CO cells. To evaluate mitochondrial function, we measured COX activity as a marker of the respiratory chain. In these experiments, we found that incubation of the cells for 2 hours in the presence of CORT (CORT-2) significantly decreased the activity of this enzyme, which continues to decrease after the removal of CORT from the culture media, as seen in the WCORT-24 cells. When we included IGF-II in the culture medium, the COX activity was significantly higher than that seen in WCORT-24 although lower than that seen in CO cells. The addition of JB1 to the culture media did not modify the behaviour of IGF-II (Figure 2g).

Incubation of control cells with IGF-II during 22 hours did not produce any change in mitochondrial function marker (Figure S2b).

In order to determine the duration of the effect of IGF-II on mitochondrial function, we resort to the same model described in 3.1 (and Figure S3a). In the first situation, CORT-2+IGF-II-2 group, the results obtained showed that IGF-II decreases COX activity compared with CO reaching values similar to CORT-2 group. This decrease in COX activity was amplified when IGF-II was removed from the media and the

incubation maintained for 20 more hours (WCORT-24+IGF-II-2) ( $p < 0.05$  vs CORT-2+IGF-II-2), with similar values than those found when no IGF-II was added to the media (WCORT-24), as shown in the second situation. Conversely, this effect on COX activity was inverted when IGF-II was present on the media during the 22 hour of total incubation time, recovering values similar to control cells (CO) (Figure S3e).

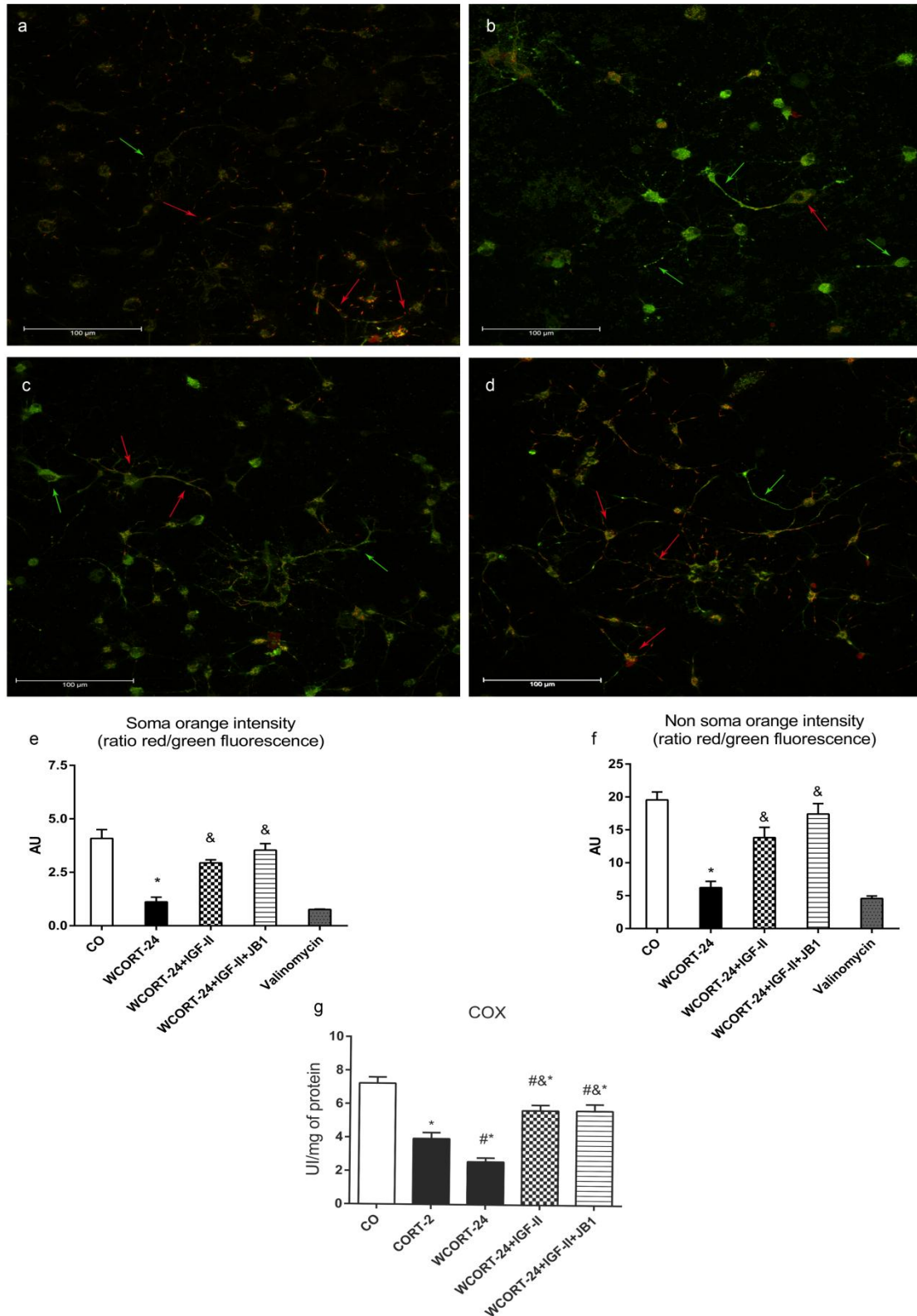




Figure 2. IGF-II restores mitochondrial function, including MMP/distribution and COX activity in neurons treated with CORT. Panels a, b, c and d represent the confocal microscopy images corresponding to JC1 staining in CO cells (a), WCORT-24 cells (b), WCORT-24+IGF-II cells (c) and WCORT-24+IGF-II+JB1 cells (d); green arrows indicate less polarized mitochondria, red arrows indicate more polarized mitochondria. Panel e represents the MMP in the soma and panel f represents depolarized mitochondria in non soma compartment, valinomycin was used as a control to completely collapse MMP. Panel g represents the COX activity in neuronal cells. Data on panel e, f and g represent mean $\pm$ SEM from 3-4 independent experiments. (\*  $p < 0.05$  versus CO; &  $p < 0.05$  versus WCORT-24; #  $p < 0.05$  versus CORT-2).

### 3.3. IGF-II regulates synapse: protein expression and function

To investigate the effects of transient high doses of CORT on the pre- and post-synaptic sites, neurons were incubated in presence of CORT for 2 hours; after that, the medium was removed, and cells were maintained for an additional 22 hours in Neurobasal® alone or supplemented with IGF-II or IGF-II+JB1.

To study pre- and post-synaptic protein expression, we performed western blot experiments and immunocytochemical analysis for synaptophysin (SYP) and PSD-95 (Figure 3a and b-e), respectively. In these experiments, we found a statistically significant decrease in the expression of both SYP and PSD-95 in WCORT-24 versus CO cells, showing a decrease in the accumulation of these proteins, whereas the expression of both were significantly increased in the WCORT-24+IGF-II versus WCORT-24 cells (Figure 3a and f). In the case of PSD-95, the levels were similar to those found in CO cells (Figure 3f), and the addition of JB1 to the culture medium did not modify the effect of IGF-II.

To explore whether the observed changes affect synaptic functions in this experimental model, we employed the FM®1-43 fluorescent probe, which is normally used to study endocytosis and exocytosis. Endocytosis is monitored by dye uptake and the subsequent increase in neuronal fluorescent intensity; conversely, exocytosis is examined by dye release and the subsequent decrease in fluorescent intensity. The dye uptake and release in these cells are shown in Figure 4a-b and in the supplementary material (Figure S5). Treatment of cells with 10  $\mu$ M CORT (WCORT-24 group) induced a significant decrease in dye uptake compared to CO cells, indicating that endocytosis in neuronal cells was decreased. The inclusion of IGF-II in the culture medium (WCORT-24+IGF-II group) induced a significant recovery in endocytosis

function as seen by the increase in dye uptake compared to WCORT-24. Again, the addition of JB1 to the culture medium did not exert any changes in the effect of IGF-II, Figure 4 (and Figure S5).

When we analysed exocytosis process, we observed a dramatic decrease in the rate of release of the FM®1-43 dye in the WCORT-24 *versus* CO cells (Figure 4a,c and Figure S5). Once more, inclusion of IGF-II in the culture medium after CORT treatment (WCORT-24 group) significantly increased the dye rate of release. The addition of JB1 to the culture medium did not exert any changes in the effect of IGF-II on the dye release.

Incubation of control cells with IGF-II during 22 hours did not produce any change in synaptic protein expression (Figure S4a) and function (Figure S4b).

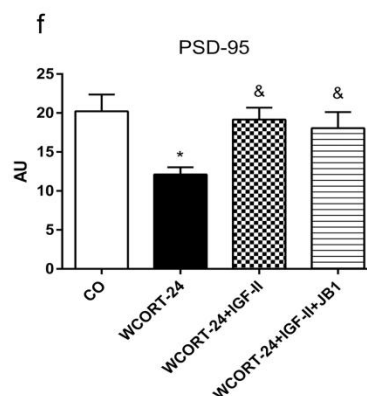
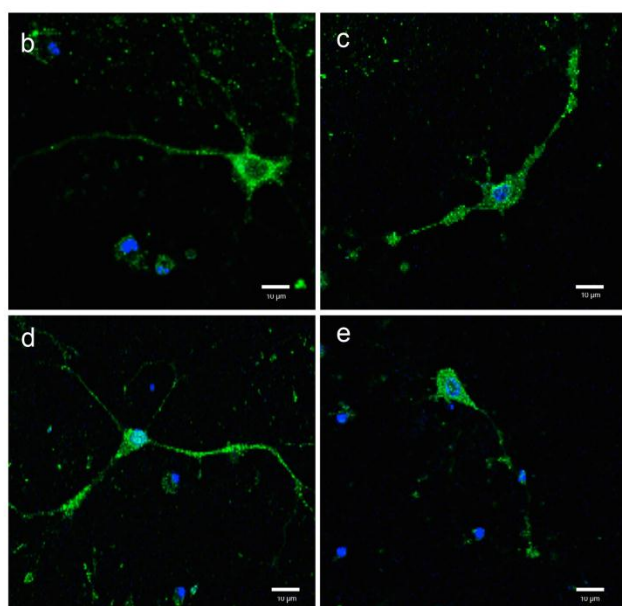
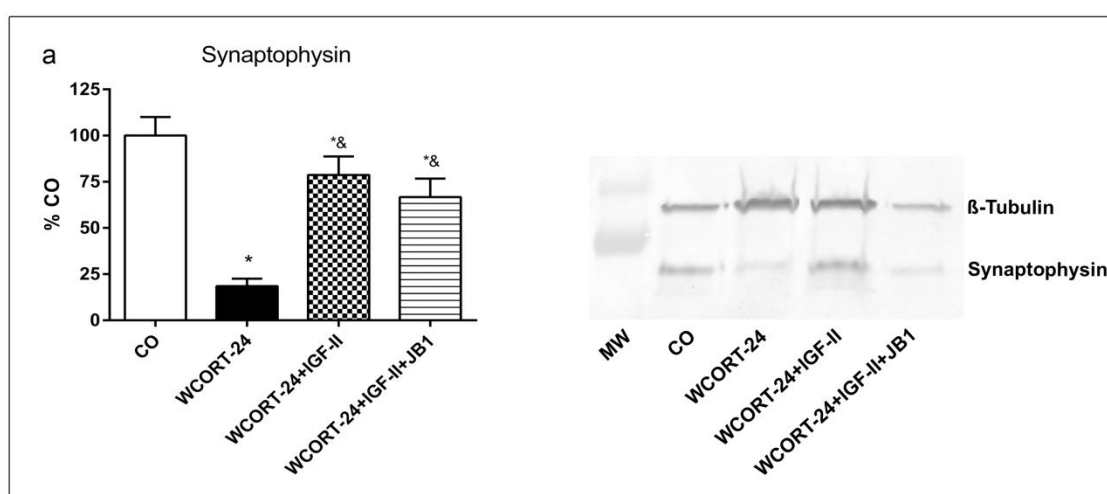


Figure 3. IGF-II modulates synaptic protein expression. Panel a shows the modulation of the expression of synaptophysin by IGF-II; quantified data is shown on the left.

Panels b, c, d and e represent the confocal microscopy images corresponding to the immunocytochemistry of PSD-95 in CO cells (b), WCORT-24 cells (c), WCORT-24+IGF-II cells (d) and WCORT-24+IGF-II+JB1 cells (e). Panel f shows the quantitative analysis of immunocytochemistry of PSD-95. Quantitative data represent the mean $\pm$ SEM from 3-4 independent experiments. (\*  $p < 0.05$  versus CO; &  $p < 0.05$  versus WCORT-24).

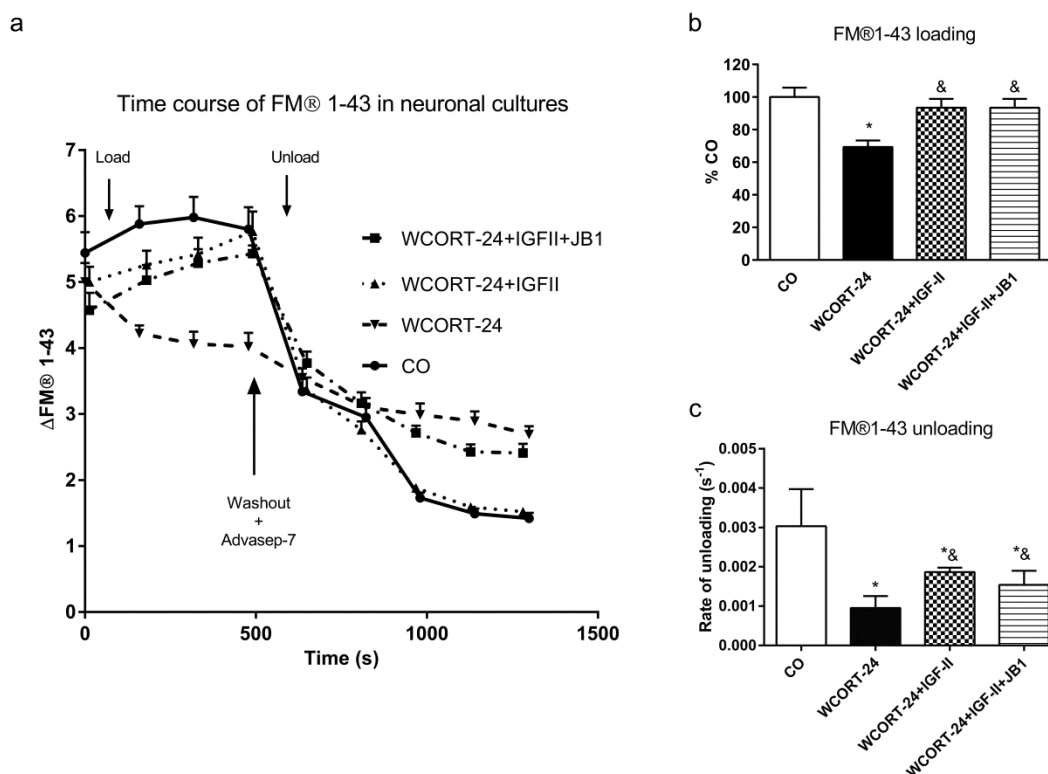


Figure 4. IGF-II modulates synaptic function. Figure a show the time course of FM®1-43 loading/unloading used to study endocytosis and exocytosis. The loading process is monitored by dye uptake and the subsequent increase in neuronal fluorescent intensity (b); conversely exocytosis is examined by dye release and the subsequent decrease in fluorescent intensity (c represents the  $1/t^{1/2}$  of unloading process). Quantitative data represent the mean $\pm$ SEM from 3-4 independent experiments. (\*  $p < 0.05$  versus CO; &  $p < 0.05$  versus WCORT-24).

### 3.4. IGF-II protects against neuronal degeneration

Neurodegeneration in neurodegenerative diseases is characterized by an increase in oxidative stress and mitochondrial dysfunction. To detect neurodegeneration independently of the mode of insult, we used the polyanionic stain FJ, a specific probe of a late stage of the degenerative processes [54]. Unfortunately, our model did not allow us to study prolonged incubation periods, as adult neuronal cultures require

antioxidants (B27® supplement) in the culture media. Thus, only short periods (24–48 h) in the absence of antioxidants can be used to avoid spontaneous degeneration of the neurons. In these experiments, a large and significant increase in the FJ fluorescence intensity in the WCORT-24 group cells was detected compared to the CO cells, but when IGF-II (100 ng/mL) was added to the culture medium after CORT removal, the fluorescence returned to similar levels to those found in CO cells, indicating a reversal of the damage induced by CORT. Again, the addition of JB1 to the culture medium did not induce any changes in the neuroprotective effect of IGF-II (Figure 5).

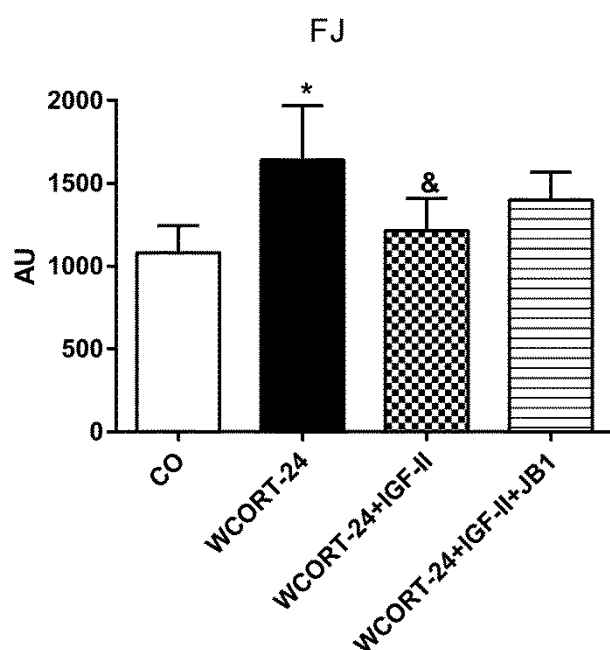


Figure 5. IGF-II improved the neurodegeneration induced by CORT measured by FJ staining. The data represent the mean $\pm$ SEM from 3-4 independent experiments. (\*  $p < 0.05$  versus CO; &  $p < 0.05$  versus WCORT-24).

#### 4. DISCUSSION

In the present study, we investigated the influence of the antioxidant effect of IGF-II-IGF1R interaction on the synaptic damage produced by transient high CORT levels. The findings described previously by our group [18] and herein show that transient high doses of CORT induce synaptic damage mediated by ROS that is maintained over time even when CORT is removed from the culture medium. This redox imbalance causes neurodegeneration with synaptic damage, including abnormal mitochondrial

membrane potential and/or distribution inside of the neuronal proteins and synapsis dysfunction. IGF-II was able to recover synaptic function by normalizing the redox balance though the interaction with IGF-IIR. This effect is supported by the increase found in cellular levels of LOOHs maintained over time compared with CO cells, which could be reverted by adding IGF-II to the culture medium. In addition, a significant decrease in antioxidant cellular TAS and NQO1 was found in these cells compared with CO. Again, TAS and NQO1 levels were recovered to CO values by the addition of IGF-II to the culture medium. The continuous presence of IGF-II in the medium is a key factor to the long lasting antioxidant protective effect of this hormone. IGF-II also recovered the expression of synaptic proteins such as SYP and PSD-95 and the dysfunctional endo/exocytosis produced by toxic levels of CORT; likewise, IGF-II recovered the neurodegeneration produced by CORT as shown in FJ dye experiments.

Increased production of reactive oxygen/nitrogen species (ROS/RNS) by high CORT levels cause oxidative/nitrosative stress [19,58,59], leading to damage in lipids, proteins, and nucleic acids [60,61]. Our results suggest that the oxidative stress induced by CORT is maintained despite CORT removal, as shown by the results of the oxidative stress markers. We previously reported that IGF-II exerts antioxidant and neuroprotective effects in different experimental models related to oxidative damage [18,62,63]. Most of these effects are more likely mediated by interactions with its specific receptor IGF-IIR, although it cannot be completely excluded that the decrease in lipid damage was also partially (nearly 39% of the effect) mediated by the IGF-IR, as shown in the brain tissue of old rats treated with IGF-I [64,65].

IGF-II restores redox imbalance, increasing the activity of NQO1 [66–68] and restoring intracellular TAS levels [18,62]. The intracellular redox balance is a key regulator of cellular energy levels, especially during brain ageing and neurodegeneration. Expression of the Nrf2-related downstream gene NQO1 decreases with age, suggesting the potential role of Nrf2 in the age-related decline of redox capacity [69]. The impairment of redox homeostasis is a hallmark of brain ageing and is particularly accentuated in the early stages of neurodegenerative diseases[70,71]. IGF-II showed effect on both TAS and NQO1. In these experiments, we found that the effect of IGF-II was again mainly mediated through interaction with its specific receptor IGF-IIR, although a small part (<15%) of the effect could be mediated by other receptors [45,64,72–74].

Activation of oxidative stress-related pathways resulting from high glucocorticoid levels, traumatic brain injuries, some neuropsychiatry diseases such as schizophrenia,

neurodegeneration and/or ageing have been implicated in mitochondrial dysfunction [75–79]. As shown in previous works, high levels of CORT induce mitochondrial damage, transforming this organelle into a source of pathological ROS [18,80,81]. This damage is maintained over time, making mitochondria a source of ROS, and COX activity remains decreased despite the removal of CORT from the incubation medium. Thus, CORT causes a bioenergetic deficit that produces MMP changes and calcium buffering modification, leading to synaptic damage and neurodegeneration [81–83]. The JC-1 fluorescence analysis performed in this study revealed a decrease in orange/red fluorescence ratio (more polarized) in both, non soma and soma compartments. These outcomes may reveal abnormalities in MMP and mitochondrial distribution, which could indicate that damaged mitochondria return to the soma, possibly for repair or destruction [24,84,85]. Based on these data, we assumed that mitochondrial trafficking to synaptic regions would be impaired by CORT treatment; this, along with the increase in ROS production, would contribute to damage in the synaptic function and axonal degeneration [31,86].

When IGF-II was added after CORT removal, we found an increase in the number of mitochondria with high MMP in the not soma compartment, reflected by the increase in orange/red fluorescence intensity compared with WCOR-24 cells. Conversely, in the soma compartment, we found a decrease in the number of mitochondria with low MMP, reflected by the decrease in green fluorescence intensity compared with the same group.

Similar results were seen in the analysis of COX activity, where CORT induced a dramatic decrease even 22 hours after removal of CORT from the medium. This decrease was almost totally reverted to CO levels by the addition of IGF-II to the culture medium. This effect is mainly due to the interaction of IGF-II with its specific receptor IGF-IIR as could not be diminished or abolished by blocking the IGF-IR.

Taking the abovementioned findings into consideration, we suggest that IGF-II in neuronal cells restores mitochondrial homeostasis [18,62], recovering its function and distribution in subcellular compartments, which are essential to the correcting the generation of energy, calcium mobilization, neurotransmitter release, neurogenesis and apoptosis [87,88].

When we studied the lasting of the antioxidant effect of IGF-II we found that the presence of the substance in the incubation medium was necessary to exert this effect. When IGF-II was removed from the media after damage with CORT and cells maintained for long period of time (20 hours), IGF-II was unable to recover the damage

produced by corticoids. In these experiments, we found a decrease in TAS amount and NQO1 activity; this could be due to the consumption of these molecules to protect against oxidative damage, as seen by the decrease in LOOH levels. One explanation could be that when IGF-II is removed from the medium, as the majority of antioxidants had been consumed to protect against initial oxidative damage, cells can not recover and levels of LOOH increase; this increase disappear when cells are incubated the same time but in presence of IGF-II.

Equally, mitochondrial function, as assessed by COX activity, does not recover from damage after short incubation periods with IGF-II; in this situation, mitochondria keeps producing ROS and consuming antioxidants damaging itself and the cells [18,80,81].

As a consequence of oxidative stress induced by different stimuli, such as glucocorticoids, the function and transport of mitochondria to different synaptic regions could be impaired, thus decreasing synaptic function and plasticity [89,90], which may result in neurodegeneration. The fluorescent probe FM®1-43 has been extensively used as a direct index of presynaptic function to study vesicle turnover for live-cell imaging [91,92], allowing the study of the processes of exocytosis in addition to endocytosis. Glucocorticoids have been reported to impair neurotransmitter release, synaptic plasticity and cognition [52,93,94]. In our study, CORT produced a decrease in the uptake and release of FM®1-43 in agreement with data reported by Muller et al [93], who found a decrease in evoked FM®1-43 destaining associated with a decrease in neurotransmitter release. IGF-II seems to partially recover exocytosis, whereas it totally recovers endocytosis. This effect is again mediated by interaction with its specific IGF-IIIR, which was not abolished by blocking the IGF-IR by the specific antagonist JB1. The findings of our work support that IGF-II promotes changes in synaptic function and/or neurotransmitter modulation related with plasticity, in accordance with data from other authors who found an improvement in memory and cognition processes along with an increase in neurotransmitter release after treatment with IGF-II [43,95–99].

To complement the synaptic study, we investigated the molecular components of pre- and post-synaptic compartments using SYP and PSD-95, respectively. SYP is a protein located at synaptic vesicles that is involved in synapse formation, neurotransmitter release and learning and memory processes [100,101].

Oxidative damage and/or high concentration of corticoids induce decreases in SYP, along with structural remodelling of neurons with synaptic loss [36]. In our work, we observed that CORT produces a decrease in the amount of the nerve terminal SYP

protein, which is related with an increase in oxidative damage, as has been reported previously by other authors [102–104]. The addition of IGF-II to the incubation medium reverted the damage produced by CORT, recovering SYP level to 78.7% compared to CO cells. The effect of IGF-II is mainly due to the interaction with its specific IGF-IIR, as the addition of the IGF-IR antagonist JB1 did not induce significant differences in the effect of IGF-II. Taken together, the levels of SYP and evoked FM@1-43 destaining after treatment with IGF-II suggest a recovery in the mechanisms of synaptic plasticity involved in memory and learning processes. This is in agreement with the neuroprotective mechanism described for other neuroprotective compounds [105,106].

Several authors have proposed PSD-95, a critical scaffolding component, as a core protein related with postsynaptic machinery needed for synaptic function and plasticity [107,108]. In our work, we observed that CORT induces a decrease in the amount of PSD-95, which is related with an increase in oxidative damage, in agreement with other authors [32,56,109]. In our study, this protein displayed similar behaviour as that found for SYP, with decreased levels in CORT-treated cells and recovery after treatment with IGF-II. Again, this effect could not be reversed when the IGF-IR was blocked, supporting the idea that it is mainly mediated by the IGF-IIR. Increases in both SYP and PSD-95 by IGF-II treatment may indicate the recovery of synaptic contact and connection circuits in cultured cells under oxidative stress induced by CORT. Related with this, Schmeiser et al. [11] showed that IGF-IIR was enriched at post-synaptic sites, indicating that IGF-II/IGF-IIR are important for synapse formation, maturation and mechanisms required for synaptic plasticity in primary embryonic cultures. In order to assess whether the effect was due to the recovery of the neuronal function or to an effect of IGF-II promoting synaptic growth, control cells untreated with corticoids were exposed to IGF-II in the same conditions than treated cells. In these experiments we did not find any statistically significant differences in either expression or function of synaptic proteins. Therefore, in our experimental model using SYP and PSD-95 as synaptic markers, IGF-II by itself has not a direct effect on synaptogenesis of non-damaged cells. These outcomes suggest that the IGF-II recovery of the synaptic impairment may be due, at least in part, to the reversion of the oxidative damage induced by CORT. In addition, in different models of oxidative damage, such as Alzheimer's disease or ageing, several authors have shown a direct relation between IGF-II administration and rescue of the synaptic dysfunction involved in learning and memory deficits produced in these models [12,110].

Neuronal treatment with CORT triggers the oxidative-mediated mechanism that impacts molecular components of pre- and post-synaptic compartments, inducing



changes in mitochondrial MMP/distribution, the recycling of synaptic vesicles and ultimately the function of synapses, which may result in neurodegeneration, as observed by FJ staining experiments [18,89,111,112] .

In conclusion, treatment of cells with IGF-II recovers the damage produced by CORT, restoring synaptic function and decreasing neurodegeneration. These outcomes can be attributed to an antioxidant effect mediated by the interaction of IGF-II with its specific IGF-IIR, which in turn mediates recovery of the redox balance via inhibition of ROS production, improvement of mitochondrial MMP/distribution and/or regulation of synaptic proteins [18,75,109], although we cannot rule out other mechanisms [9,12,16,17,112–114].

From our results, we may conclude that IGF-II treatment exerts an antioxidant effect that can be considered as a potential tool to reduce or revert the neuronal damage, making this hormone a potential therapeutic agent in the treatment of neurodegenerative diseases mediated by oxidative damage, such as Parkinson's disease, Alzheimer's disease, hypoxia or traumatic brain damage. Moreover, the neuroprotective effect of IGF-II would be especially useful in ageing and pathologies associated with cognitive deficits and/or neuropsychiatric diseases.

#### ***Competing financial interests statement and source of funding***

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## HIGHLIGHTS

First evidence that IGF-II reverts oxidative synaptic damage produced by corticoids.  
 IGF-II recovers mitochondrial function in synapses after oxidative damage.  
 IGF-II restores mitochondrial distribution in neurons after oxidative damage.

Evidence of the involvement of IGF-II receptor in the recovery of synaptic function.

IGF-II reverts neurodegeneration induced by oxidative damage produced by corticoids.

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