RESEARCH ARTICLE

Astrocytes require insulin-like growth factor I to protect neurons against oxidative injury [v1; ref status: indexed, http://f1000r.es/2lf]

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Abstract

Oxidative stress is a proposed mechanism in brain aging, making the study of its regulatory processes an important aspect of current neurobiological research. In this regard, the role of the aging regulator insulin-like growth factor I (IGF-I) in brain responses to oxidative stress remains elusive as both beneficial and detrimental actions have been ascribed to this growth factor. Because astrocytes protect neurons against oxidative injury, we explored whether IGF-I participates in astrocyte neuroprotection and found that blockade of the IGF-I receptor in astrocytes abrogated their rescuing effect on neurons. The protection mediated by IGF-I against oxidative stress (H_2O_2) in astrocytes is probably needed for these cells to provide adequate neuroprotection. Indeed, in astrocytes but not in neurons, IGF-I helps decrease the pro-oxidant protein thioredoxin-interacting protein 1 and normalizes the levels of reactive oxygen species. Furthermore, IGF-I cooperates with trophic signals produced by astrocytes in response to H_2O_2 such as stem cell factor (SCF) to protect neurons against oxidative insult. After stroke, a condition associated with brain aging where oxidative injury affects peri-infarcted regions, a simultaneous increase in SCF and IGF-I expression was found in the cortex, suggesting that a similar cooperative response takes place *in vivo*. Cell-specific modulation by IGF-I of brain responses to oxidative stress may contribute in clarifying the role of IGF-I in brain aging.

Article Status Summary

Referee Responses

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Introduction

Oxidative stress is usually considered a mechanism of brain aging[1]. However, contradictory data[2] and lack of firm evidence[3] makes it difficult to firmly establish its actual significance in this process (see López-Otín et al.[4] for a recent review). One important aspect that requires further clarification in this regard is the relationship between oxidative stress and insulin peptides, a well conserved family of hormones firmly linked to aging. Extensive work in vertebrates and invertebrates indicates that the insulin-like growth factor I (IGF-I)/insulin signalling (IIS) pathway has a negative impact on aging. It has been argued that this detrimental action is mediated by reducing cell defences to oxidative stress[5–7] which, in turn is harmful for neuronal survival[1]. However, IGF-I has been shown to be largely neuroprotective[8], even in conditions such as ischemic injury or brain trauma where oxidative stress is most likely a major pathogenic mechanism[9]. Thus, it is unclear whether or not IGF-I protects the brain against oxidative stress as the current evidence is contradictory.

A possible explanation for these apparently contradictory observations may be that modulation of the cellular response to oxidative stress by IGF-I is cell-dependent[10]. Until now, only neurons have been studied in this regard. However, astrocytes, a major cellular element of the brain, are essential contributors to neuronal homeostasis and are coupled to neurons in the response to oxidative stress in order to help protect them[11]. It is thus possible that IGF-I participates in the response of astrocytes to oxidative stress as part of the overall brain response. Contrary to what we previously observed in neurons[12], we report here that IGF-I protects astrocytes against oxidative stress and, very significantly, also co-operates with astrocytes to protect neurons.

Methods

Animals

We used postnatal rats and mice for in vitro cultures (P0-3 days for astrocytes and P7 for neurons) and 3 month old mice for in vivo experiments. P2 Wistar rats (8 g ± 0.04 body weight, n=240, Harlan, Spain), P3 (2 g ± 0.03, n=36, Harlan) and 3 months old (27.6 g ± 0.812; n= 24) C57BL6 mice and P7 GFP transgenic mouse pups (4.25 g ± 0.22, n=126; in-house colony) were used. Pups used were of both sexes and no attempt to sex them was done. Adult mice were male. Rat tissue was used in all in vitro experiments except when using GFP cells derived from transgenic mice. In vivo experiments were done in mice for future comparisons with transgenic mice. All efforts were made to minimize suffering and reduce the number of animals. Animals were kept under light/dark, (12h/12h) conditions following EU guidelines (directive 86/609/EEC) and handled according to institutionally-approved procedures (CSIC bio-ethics subcommittee; project code SAF2010-1703). Animals were fed ad libitum with laboratory rodent chow (Teklad Global 2018S) and kept in standard laboratory cage conditions with 4 animals/cage.

Reagents

Antibodies used in this study are detailed in Table 1. The different drug inhibitors used in the study are given in Table 2. Hydrogen peroxide (H_2O_2) and the calcium chelator BAPTA-AM were purchased from Sigma (Steinheim, Germany). IGF-I and SCF were purchased from Prospec-Tany Technogene, (Israel).

Plasmids

pECE-FOXO3 and pECE-FOXO3-TM (triple mutant T32A/ S253A/S315A, herein called MFOXO3) were kindly provided by ME Greenberg (Harvard Medical School, Boston, USA). p6xDBE-luc (reporter luciferase plasmid with six copies of the DAF16 family protein-binding element) and pRL-TK (TK-Renilla luciferase) were a kind gift of BM Burgering (University Medical Centre, Utrecht, The Netherlands). Dominant negative IGF-IR expression plasmid was kindly donated by D. Le Roith (Mt Sinai, New York, USA). Plasmids expressing shRNA for TXNIP1 were purchased from Origene (USA). Txnip1 plasmid was purchased from Thermo Scientific Open Biosystems (Waltham, USA).

Cell culture and transfections

Cerebellar granule cultures were produced from either P7 rat or GFP transgenic mouse cerebella as previously described[13]. In brief, cells were plated onto 6 or 12-well dishes coated with poly-L-lysine (1 μg/ml) at a respective final density of 1.5×10^6/well or 0.45×10^6/ well. Cells were incubated at 37°C/5% CO_2 in Neurobasal (Gibco, USA) medium supplemented with 10% B27 (Gibco), glutamine (5 mM) and KCl (25 mM). All experiments were carried out in 2–7 day old cultures, with neurons showing neurite extensions. Different times in vitro were used to analyze time-dependent parameters such as cell survival. On the day of the experiment, medium was replaced with Neurobasal + 25 mM KCl. Two hours later, IGF-I (10^{-7} M) and/or hydrogen peroxide (H_2O_2) at doses of 50–150 μM were added. Inhibitory drugs were given 45 min before treatments. We used H_2O_2 as an oxidant stimulus because it is an endogenously produced reactive oxygen species (ROS) that serves as a precursor to hydroxyl radicals and possesses signalling capacities[14]. Astrocyte cultures were prepared from P3 rat or GFP mouse forebrain, as previously described[15] after animals were sacrificed by decapitation. Cells were grown on Dulbecco's modified Eagle's medium F12 (DMEM-F12) supplemented with 10% fetal calf serum. After 12 days astrocytes were seeded at 2.5×10^5 or 1.25×10^5 cells/well in 6-well and 12-well culture plates, respectively. On the day of the experiment cells were treated with IGF-I (10^{-7}M), H_2O_2 (50–200 μM) and/or inhibitors, as above. For transfection, astrocytes were seeded at 2.5×10^5 or 1.25×10^5 cells/well in 6-well and 12-well culture plates respectively, and after 16h constructs were mixed with Fugene HD (Roche, Switzerland) in a 1:3 ratio, and added following the manufacturer's instructions. Alternatively, astrocytes were electroporated (2×10^6 astrocytes with 2 μg DNA or shRNA) before seeding using an astrocyte Nucleofector Kit (Lonza, Switzerland). After electroporation, cells were plated to obtain a final cell density on the day of the experiment similar to that obtained with the transfection method. All experiments were performed after 48h. The transfection efficiency was 20–30% and 60–80% for electroporation, as assessed with a GFP vector. At least three independent experiments were done in duplicate wells.

Cell assays

Cell viability was determined by different methods. When using transfected astrocytes, a GFP-pCMV vector and the different

Table 1. Antibodies used in the study.

ANTIBODY	PRODUCT Nº	MANUFACTURER	WORKING CC	SPECIES	ISOTYPE	ANTIGEN (EPITOPE)	AFFINITY PURIFIED	REFERENCE
Akt1/2 (H-136)	sc-8312	Santa Cruz Biotechnology (California, USA)	1:1000	rabbit	polyclonal	aminoacids 345–480 of human Akt1/2	unknown	42–45
β-actin (Clone AC-74)	A5316	Sigma (Steinheim, Germany)	1:50000	mouse	monoclonal	N-terminal end of β-isoform of actin	ascytes fluid	46–49
Cu/Zn superoxide dismutase (SOD)	SOD-101	Assay Designs (Michigan, USA)	1:1000	rabbit	polyclonal	Native rat Cu/Zn SOD	yes	50,51
MnSOD superoxide dismutase (SOD)	SOD-111	Assay Designs (Michigan, USA)	1:2500	rabbit	polyclonal	Native rat Mn SOD	yes	52–55
p44/p42 MAPK (ERK1/2)	9102	Cell Signalling (Danvers, USA)	1:2000	rabbit	polyclonal	sequence in the C-terminal of rat p44MAPK	yes	56–59
phospho-Akt (Ser473)	9271	Cell Signalling (Danvers, USA)	1:1000	rabbit	polyclonal	residues surrounding Ser473 of mouse Akt	yes	46,60–63
phospho-ERK1/2 (Thr202/Tyr204)	9101	Cell Signalling (Danvers, USA)	1:2000	rabbit	polyclonal	residues surrounding Thr202/Tyr204 of human p44 MAPK	yes	63,63,63–66
SCF	sc-9132	Santa Cruz Biotechnology (California, USA)	1:1000	rabbit	polyclonal	aminoacids 26–214 of human SCF	unknown	67–69
TXNIP1	K0205-3	MBL (Nagoya, Japan)	1:2000	mouse	monoclonal	human recombinat TXNIP	unknown	no refs

constructs under evaluation were used in a 1:5 ratio. In this case, GFP$^+$ astrocytes were scored prior to treatment to determine baseline survival (time 0) and at different times as indicated in the results. Alternative viability assays for astrocytes included measuring cell metabolism with fluorescein diacetate (0.1 µg/ml FDA) or number of propidium iodide (PI) cells[12] as specified in the results section. For the latter, cells were stained with 2 µg/ml PI as a marker of dead cells plus DAPI staining as a marker of total cell number. PI$^+$ and DAPI$^+$ cells were counted under a Leica CTR 6000 fluorescence microscope. Percentage of viable cells indicates the number of PI$^+$ cells related to total cell number. The experiments were done in triplicate and a total of three independent experiments were done. For neuronal-specific viability assays, cerebellar neurons from GFP mice were seeded on 12-well plates (4.5×10^5 cells) coated with poly-L-Lysine and grown with Neurobasal medium plus B27, 4 mM glutamine and 25 mM KCl. After 4–5 days, cultures were treated with 100 nM IGF-I in the presence or

absence of 50–100 µM H_2O_2. Pictures of GFP$^+$ cells (green fluorescence) were taken every 24 hours up to 3 days in an Incucyte™ 2010A Rev2 system (Essen BioScience, USA). For co-cultures, 1.25×10^5 wild type mouse astrocytes/well were seeded on 12-well plates and grown with DMEM-F12 plus 10% FBS. After 48–72 hours, GFP neurons were isolated and plated onto astrocytes. We used forebrain astrocytes and cerebellar neurons because in our experience the forebrain and cerebellum yielded very high numbers of astrocytes and neurons, respectively (thus minimizing animal use). Furthermore, in this study we were interested in exploring general, rather than region-specific neuroprotective characteristics of astrocytes. Nevertheless, we also carried out co-cultures with neurons and astrocytes from the same region (forebrain) and the results obtained were identical than when using cells from differing regions (see Figure 2 in results). Culture medium was changed to DMEM-F12 plus B27, 4 mM glutamine and 25 mM KCl (the latter only in the case of neurons). Two days later, co-cultures were treated with

Table 2. Drug inhibitors used in the study.

Target	Inhibitor	Dose	Supplier
CALCINEURIN	CYCLOSPORIN A	500 nM	Sigma-Aldrich
ERK MAPK	U0126	20 µM	Tocris Bioscience
Extracellular Ca2+	CdCl2/EGTA	100 µM/10 mM	Sigma-Aldrich
IGF-IR	PPP	120 nM	Calbiochem
Intracellular Ca_{2+}	BAPTA/AM	5–10 µM	Calbiochem
JNK	Insolution™ JNK INHIBITOR II	10–20 µM	Calbiochem
mTOR	Insolution™ RAPAMYCIN	100 nM	Calbiochem
NF-KB	QNZ	10–20 nM	Enzo Life Sciences
p38 MAPK	SB203580 hydrochloride	20 µM	Calbiochem
PDK1	OSU-03012	10 µM	Echelon
PI3K	LY294002	25 µM	Calbiochem
PKA	KT5720	60 nM	Tocris Bioscience
PKC/PKA	Ro 31-8220	20–900 nM	Calbiochem
PKCα, PKCβI, PKCε	Rho 32-0432	0.2 µM	Calbiochem
PKC isotypes (α,β,γ,δ,ζ,μ)	Go6983	6 nM–20 µM	Tocris Bioscience
PP1	TAUTOMYCIN	2 nM	Calbiochem
PP2A	OKADAIC ACID (495609 Insolution)	2.5 nM	Calbiochem
PROTEASOME	MG-132	5 nM–3 µM	Calbiochem
PROTEIN SYNTHESIS	CYCLOHEXIMIDE	1 µg/ml	Calbiochem

100 nM IGF-I $\pm$ 50–100 µM H_2O_2. Pictures were taken every 24 hours up to 5–7 days as above. For protein silencing or overexpression, 2×10^6 astrocytes were electroporated in a Nucleofector®II (Amaxa Biosystems Lonza, Switzerland) and seeded at 1.25×10^5/well. Co-cultured neurons were seeded as described above. Viability of neurons was assessed by counting the number of cells expressing GFP using Incucyte software with a set cell size threshold to avoid including GFP$^+$ cell debris and dying cells. Viability is expressed as percentage of GFP$^+$ cells at the beginning of the experiment (time 0). At least three independent experiments were done.

Immunoassays
Western blotting was performed as described[13]. Neurons were washed once with ice-cold PBS and lysed with 1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4, 10% glycerol, 1 mM CaCl2, 1 mM $MgCl_2$, 400 µM sodium vanadate, 0.2 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 0.1% phosphatase inhibitor cocktails I and II (Sigma-Aldrich). To normalize for protein load, membranes were reblotted (Re-Blot, Chemicon, USA) and incubated with an appropriate control antibody (see Results). Levels of the protein under study were expressed relative to protein load. Different exposures of each blot were collected to ensure linearity and to match control levels for quantification.

Densitometric analysis was performed using Analysis Image Program (Bio-Rad, USA). A representative blot is shown from a total of at least three independent experiments. IGF-I levels in culture medium were measured using Quantikine ELISA for mouse/rat IGF-1 (R&D Systems, USA). In brief, cells were treated as described above and 1 ml of culture medium was collected after 24 hours, spun to eliminate cell debris, and stored at -80°C. Samples were lyophilized overnight and resuspended in 150 µl of calibrator buffer. After vortexing, samples were centrifuged 10 min/14,000 rpm (Hettich, Germany) and assayed according to manufacturer´s instructions. A total of three and four independent experiments were done for neurons and astrocytes, respectively.

Luciferase assays
Luciferase assays were done as previously reported[12]. In brief, cells were transfected with a reporter construct bearing six canonical FOXO binding sites (6×DBE-luciferase) and co-transfected with different constructs, as indicated in each experiment. Transfections were performed in triplicate dishes. Luciferase counts were normalized using TK-Renilla luciferase. At given times, neurons were lysed in passive lysis buffer (PLB) and luciferase activity was analysed using a luminometer and dual luciferase assay kit according to the manufacturer (Promega, USA). Background luminescence

was subtracted. Luciferase activity was expressed as fold of increase over control levels. At least three independent experiments were done.

Flow cytometry

After 18h of exposure to H_2O_2, cell death was assessed. Cells were detached using 0.25% Trypsin-1.3 mM EDTA (Invitrogen) during 5–10 minutes, centrifuged ($200 \times g$, 5 min/4°C), and resuspended in cold PBS. Propidium iodide (PI 5 μg/ml; Sigma) in PBS was added prior to flow cytometry analysis using a FACSAria cytometer (BD Biosciences). Fluorescence intensity, forward scatter (FSC), and side scatter (SSC) were collected in logarithmic scale. The emission filter used was 600–620 nm band pass (FL3). A fluorescence blank was measured and subtracted from the fluorescence of the sample. Dead cells were identified as red fluorescence positive events with low FSC (small PI permeable cells). Debris was always excluded from the analysis. At least three independent experiments were conducted.

ROS measurement

Mitochondrial O_2- production levels were measured by using the fluorescent probe MitoSOX™ Red (Life Technologies, USA). Briefly, astrocytes were pre-treated overnight with IGF-I and then 200 μM H_2O_2 were added during 1 hour. Cells were incubated with 1.5 μM MitoSOX™ Red in DMEM-F12 for 10 min/37°C and washed 3 times with PBS. Astrocytes were then trypsinized and fluorescence was measured by flow cytometry (510 nm excitation/580 nm emission) using the cytometer, as described[15]. A total of six independent experiments were done. Alternatively, ROS generation was assessed in astrocytes cultured on coverslips with the fluorogenic marker carboxy-H_2DCFDA (Molecular Probes, USA) during 30 min/37°C, protected from the light. When using this ROS marker it is not possible to distinguish endogenous ROS from exogenously applied H_2O_2. Nevertheless, we compared this method to the oxidation of luminol (which detects superoxide anions) that distinguishes H_2O_2 from other ROS and we obtained identical results with either method (data not visualized). The reason we used carboxy-H_2DCFDA is because we could obtain both qualitative (cell images) and quantitative (fluorimetry assay) measurements within the same assay. After incubation with carboxy-H_2DCFDA, cells were gently washed 3 times with warm DMEN, and mounted, or, alternatively, assayed for fluorimetry. Pictures were taken at 40× magnification using a Leica fluorescence microscope (Germany). A representative picture is shown. Fluorescence intensity in lysed cells was measured using a FluoroStar fluorimeter.

Growth factor gene array

An RT² Profiler™ PCR Array (SABiosciences, USA) was used to screen a battery of growth factors following the manufacturer´s recommendations. After treatment, astrocytes were lysed and RNA extracted using Trizol (Life Technologies, USA). The resulting cDNA synthesis reaction was diluted in water, mixed with the qPCR master mix, and loaded in a 96 well PCR Array plate. PCR was performed following manufacturer´s instructions.

Brain focal ischemia

Three-month old male mice (4–6 per group) were anesthetized with 3% isoflurane (in 70% N_2O, 30% O_2) for induction and with 2% isoflurane for maintenance. Rectal temperature was maintained at 36.5 C with a heating pad. The frontal branch of the medial cerebral artery (MCA) was exposed and occluded permanently by suture ligation as previously reported, with modifications[16]. Briefly, an incision perpendicular to the line connecting the lateral canthus of the left eye and the external auditory canal was made to expose and retract the temporalis muscle. A burr hole was drilled, and frontal and parietal branches of the MCA were exposed by cutting and retracting the dura. The frontal branch of the MCA was elevated and ligated with a suture nylon monofilament 8/0. Following ligation, a sharp decrease of blood flow was evidenced with a laser Doppler flowmetry (Järfalla, Sweden). Following surgery, mice were returned to their cages, kept at room temperature and allowed free access to food and water. All physiological parameters measured: rectal temperature, mean arterial pressure and blood glucose levels were not different between groups. Sixteen hours after medial cerebral artery occlusion (MCAO), animals were killed by neck dislocation by an experienced researcher to assess infarct outcome. The brain was removed and the infarcted area isolated and processed for RNA and protein isolation.

Quantitative PCR

Total RNA isolation from cell lysates or brain tissue was carried out with Trizol. One μg of RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. For the quantification of specific genes, total RNA was isolated and transcribed as above and 62.5 ng of cDNA was amplified using TaqMan probes for Txnip1, IGF-I or SCF and 18S as endogenous control (Life Technologies). Each sample was run in triplicate in 20 μl of reaction volume using TaqMan Universal PCR Master Mix according to the manufacturer's instructions (Life Technologies). All reactions were performed in a 7500 Real Time PCR system (Life Technologies). Quantitative real time PCR analysis was carried out as previously described[17]. Results were expressed as relative expression ratios on the basis of group means for target transcripts versus reference 18S transcript. At least three independent experiments were done.

Statistical analysis

Data are expressed as mean ± SEM. Differences among groups were analyzed by one-way ANOVA followed by a Newman-Keul's or t-test using Graph Pad Prism softwareA $p<0.05$ was considered significant.

Results

Astrocyte neuroprotection against oxidative stress requires IGF-I signalling onto astrocytes

Whereas neurons cultured without astrocytes are very sensitive to acute oxidative insult elicited by H_2O_2 (Figure 1A), when cultured with astrocytes, neurons become very resilient (Figure 1A). To

determine whether IGF-I participates in the neuroprotective effects of astrocytes against oxidative stress we first confirmed that it is endogenously produced by these cells. As shown in Figure 1B, not only astrocytes but also neurons (albeit at much lower levels) secrete IGF-I into the culture medium. In response to H_2O_2 astrocytes secrete lower, but still substantial, amounts of IGF-I, and so IGF-I may still participate in neuroprotection by astrocytes. To directly test this possibility we blocked IGF-I signalling in astrocytes with a dominant negative (DN) IGF-IR[18] (Figure 1C) and determined their ability to protect neurons against oxidative challenge. As shown in Figure 1D, a significantly greater percentage of neurons co-cultured with mock-transfected astrocytes survived compared

to neurons cultured with astrocytes transfected with DN IGF-IR. Similar results were obtained after H_2O_2 challenge (not visualized).

We next used pharmacological blockade of the IGF-I receptor using picropodophyllin (PPP), an antagonist of IGF-IR (Figure 2A). As in this case both the neuronal and astrocyte receptors are blocked, we first determined whether neurons are affected by PPP blockade of the IGF-I receptor. In the presence of H_2O_2, neurons cultured alone die regardless of the presence or absence of proper IGF-I signalling since PPP did not increase neuronal death (Figure 2B). This agrees with our previous findings that IGF-I does not protect cultured neurons against oxidative stress[12]. Confirming the results seen with
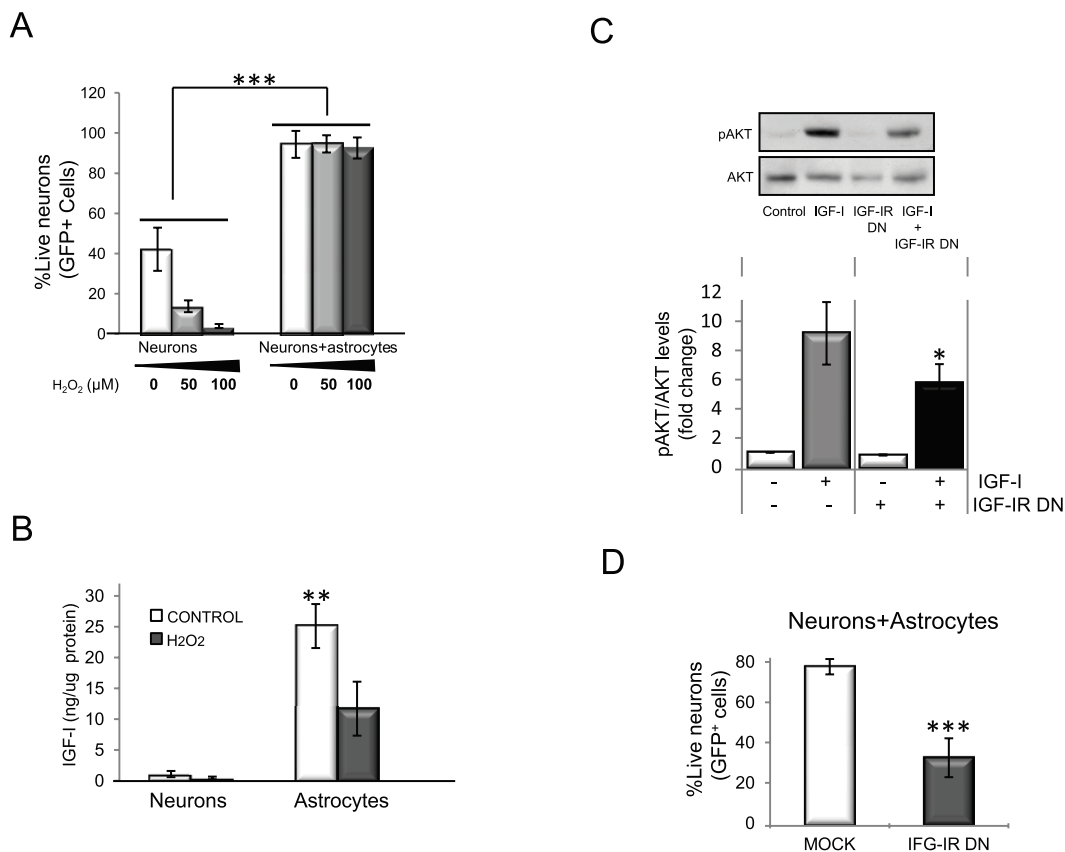

Figure 1. IGF-I signalling participates in astrocyte neuroprotection against oxidative injury. A) Neurons are protected from oxidative stress in the presence of astrocytes whereas when cultured alone they rapidly die. Viability of GFP neurons was measured as the number of green (GFP$^+$) cells two days after H_2O_2 treatment in the presence or absence of wild type astrocytes (F=41.85; ***p<0.001 vs. neurons alone). **B)** Both astrocytes and neurons secrete IGF-I, although astrocytes produce much higher levels (*p<0.05 vs. neurons). H_2O_2 lowers IGF-I secretion. **C)** Astrocytes were transfected with a dominant negative IGF-IR (IGF-IR DN) and the ratio pAkt/Akt (histograms) was measured as an index of IGF-I signalling. In the presence of DN IGF-IR, signalling by IGF-I was markedly reduced. Representative blots and quantitative histograms are shown (*p<0.05 vs. IGF-I alone). **D)** Blockade of IGF-IR function with dominant negative IGF-IR compromises neuroprotection by astrocytes. GFP neurons were seeded on top of wild type astrocytes transfected with an IGF-IR DN construct or mock-transfected (control). Viability of GFP neurons was measured after 5 days (***p<0.001 vs. control). Experiments were done at least 3 times in this and all following figures. Bars are SEM in this and following figures.
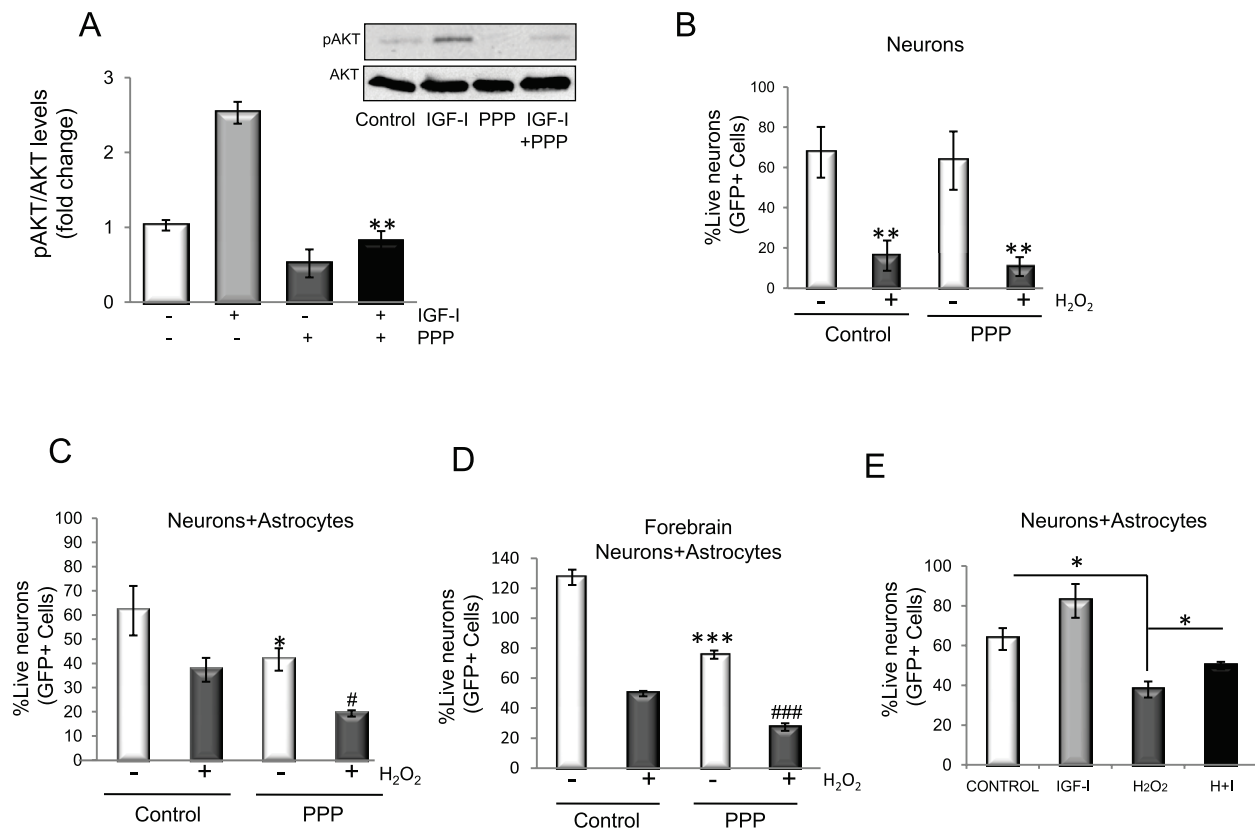
Figure 2. Endogenously produced IGF-I protects neurons against oxidative injury. A) The IGF-IR inhibitor PPP blocks IGF-I signalling in astrocytes. Astrocytes were treated with 120 nM PPP 1h before adding IGF-I while pAkt levels were measured 10 minutes after adding IGF-I. Ratios are shown in histograms (**$p<0.01$ vs. IGF-I alone). Representative blot is shown. **B**) Blockade of IGF-IR signalling with PPP in neurons cultured alone does not affect H_2O_2 toxicity after 3–4 days of exposure (F=8.124, **$p<0.01$ vs. respective controls). Note that PPP alone does not affect neuronal survival. **C**) Viability of cerebellar neurons co-cultured with forebrain astrocytes decreased significantly when treated with PPP for six days. PPP treatment in the presence of H_2O_2 decreased neuronal viability even further (F=8.90; *$p<0.05$ vs. untreated control and #$p<0.05$ vs. H_2O_2). **D**) Viability of forebrain neurons co-cultured with forebrain astrocytes decreased significantly when treated with PPP for five days. PPP treatment in the presence of H_2O_2 decreased neuronal viability even further (F=170.2, ***$p<0.01$ vs. untreated control and ###$p<0.01$ vs. H_2O_2). **E**) When co-cultured with wild type astrocytes, neuronal survival after five days of exposure to 100 µM H_2O_2 was moderately increased in the presence of 100 nM IGF-I (F=9.965; *$p<0.05$ vs. control or H_2O_2). I+H: IGF-I + H_2O_2.

astrocytes transfected with dominant negative IGF-I receptor, a reduction in neuroprotection by astrocytes was seen when co-cultured neurons were exposed to PPP. In the presence of H_2O_2 significantly fewer co-cultured neurons survived with PPP ($p<0.01$ H_2O_2+PPP vs. H_2O_2 alone; Figure 2C). To rule out region-specific actions of astrocytes on neuroprotection we then co-cultured neurons and astrocytes from the same brain region (forebrain) and treated them with PPP. As shown in Figure 2D, forebrain neurons were similarly sensitive to blockade of IGF-IR when co-cultured with forebrain astrocytes. The observation that even supra-physiological doses of IGF-I (100 nM) added to the co-cultures only produced a modest additional effect

on neuronal survival after oxidative insult confirmed the idea that endogenous IGF-I is required by astrocytes for neuroprotection (Figure 2E). Hence, endogenous production of IGF-I is necessary and sufficient to protect neurons.

IGF-I protects astrocytes against oxidative stress

IGF-I-dependent neuroprotection by astrocytes appears to also involve a direct action of IGF-I on astrocytes. Because it is known that astrocytes are more resistant to oxidative damage than neurons, we explored whether IGF-I was involved in this greater resilience. Contrary to neurons (Figure 3A), IGF-I protected astrocytes against
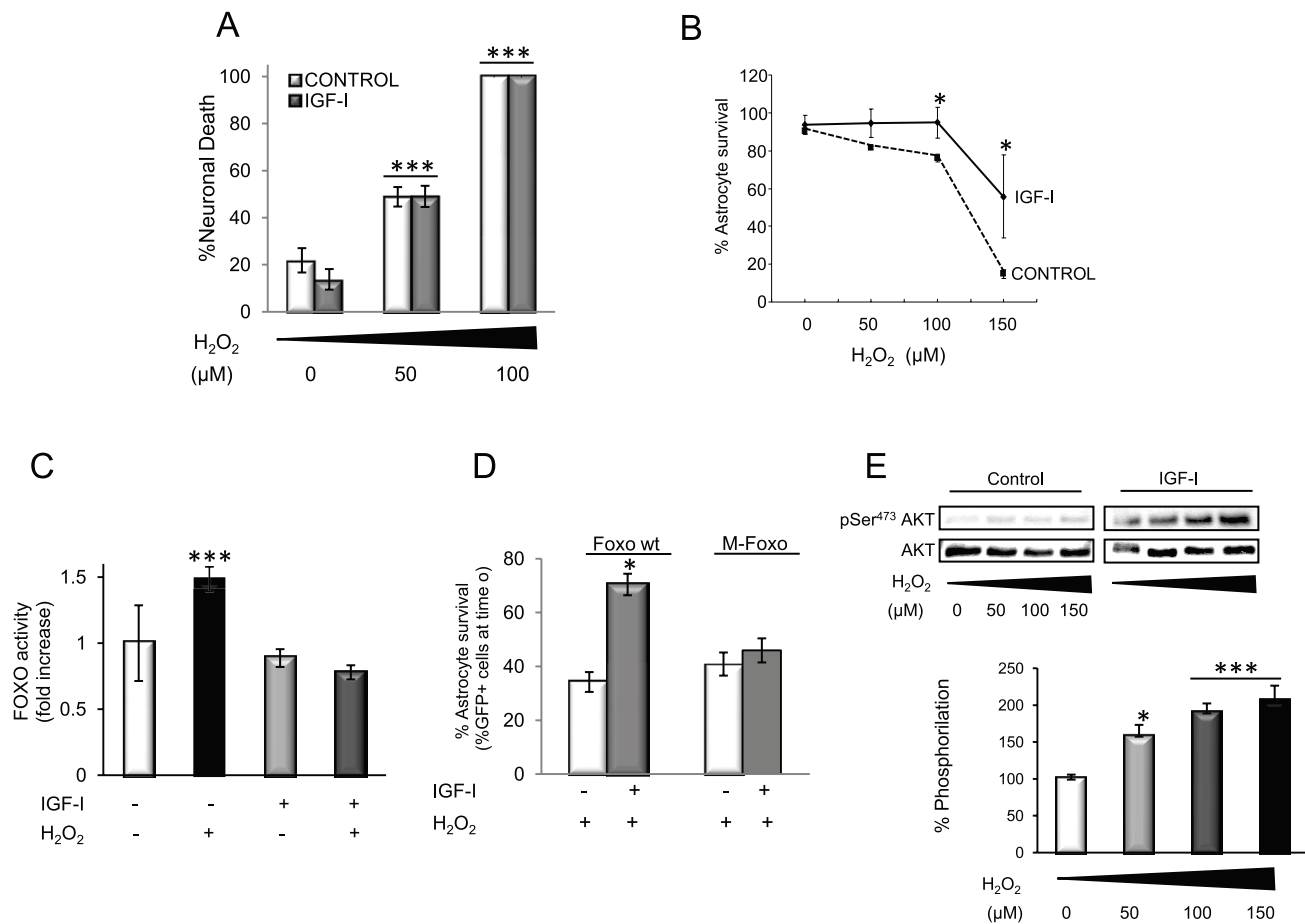
Figure 3. IGF-I protects astrocytes against oxidative stress. A) IGF-I does not rescue neurons from H_2O_2 induced death. This confirms previous observations[12]. Neuronal mortality was measured by counting PI^+ cells 6h after treatment. H_2O_2 induces neuronal death in a dose-dependent manner irrespective of the presence of IGF-I (***$p<0.001$ vs. no H_2O_2). **B**) IGF-I treatment protects astrocytes from H_2O_2 induced death. Astrocyte demise was measured by counting PI^+ cells 24h after H_2O_2 (100 μM). H_2O_2 exerts a dose-dependent effect that is reduced by IGF-I (*$p<0.05$ vs. control). **C**) IGF-I blocks FOXO activity induced by H_2O_2 (100 μM). FOXO activity was measured with a luciferase reporter in astrocytes treated with IGF-I, H_2O_2 or both for 24h (***$p<0.001$ vs. no treatment). **D**) Protection by IGF-I against death induced by H_2O_2 requires blockade of FOXO activity. Astrocyte viability was measured by counting GFP^+ astrocytes after co-transfection of GFP and a FOXO wild type (wt) or an Akt-insensitive mutant of FOXO (M-FOXO); *$p<0.05$ vs. no IGF-I. **E**) IGF-I increases phosphorylation of Akt (pAkt) in the presence of H_2O_2 in a dose-dependent fashion. Representative blots are shown. Lower histograms indicate quantification of pAkt/Akt ratio in the presence of IGF-I as shown in the right blot. pAkt levels were measured after 15 min. (*$p<0.05$ and ***$p<0.001$ vs. no H_2O_2).

H_2O_2-induced death (Figure 3B). The protective effect of IGF-I involved blockade of the activation of FOXO 3, a transcription factor involved in brain responses to oxidative stress[19], by H_2O_2 (Figure 3C). Inhibition of FOXO 3 by IGF-I was mediated by Akt; i.e.: an Akt-insensitive mutant of FOXO (M-FOXO3) abrogated IGF-I effects while wild type FOXO3 did not interfere with its protective actions (Figure 3D). Indeed, in astrocytes IGF-I activates Akt in the presence of H_2O_2 (Figure 3E), whereas in neurons H_2O_2 blocks this canonical pathway[12]. Underlying the protective actions of IGF-I on astrocytes was its ability to block excess ROS after exposure to H_2O_2 as determined by flow cytometry using MitoSOX (Figure 4A) or fluorometry with carboxy-H_2DCFDA (Figure 4B).

We then determined possible mediators of the anti-oxidative actions of IGF-I on astrocytes. We examined whether modulation of SODs could be involved because these anti-oxidant enzymes constitute an important detoxifying mechanism in cases of excess ROS. We found that cytosolic Cu/ZnSOD was increased by IGF-I, H_2O_2, or both (Figure 5A), while mitochondrial MnSOD was increased only by H_2O_2 (Figure 5B). Thus, increases in SOD levels form part of the astrocyte response to H_2O_2, and IGF-I does not appear to interfere with these enzymes. Because FOXO participates in cellular responses to ROS, we looked for signals downstream of FOXO inactivation by IGF-I such as thioredoxin inhibitor 1 (TXNIP1), a pro-apoptotic protein dependant on FOXO activity and related
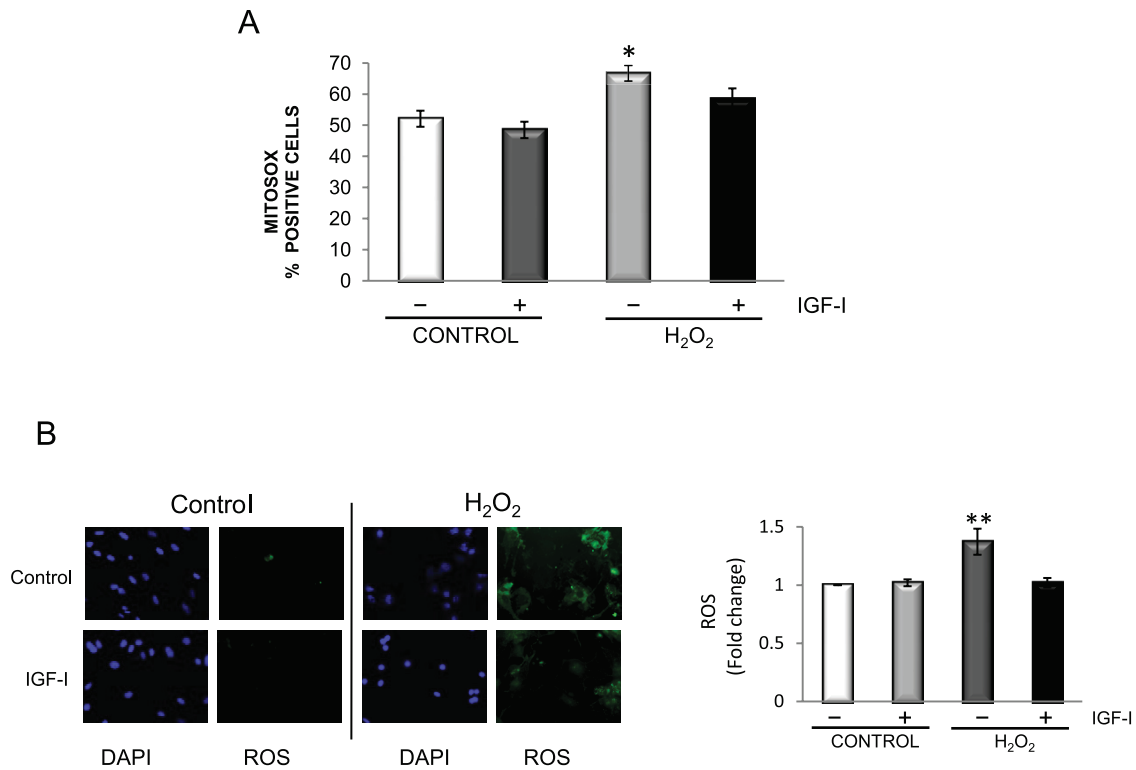
Figure 4. IGF-I reduces oxidative stress in astrocytes. A) H_2O_2 increases the number of astrocytes expressing mitochondrial O_2^-. This increase is prevented when cells are pre-treated with IGF-I. Mitochondrial O_2^- levels were detected with MitoSOX by flow cytometry. Astrocytes were treated overnight with IGF-I and for 1 hour more with 200 μM H_2O_2 (F=9.364; *p<0.005 H_2O_2 vs. control, IGF-I and IGF-I + H_2O_2). **B**) IGF-I lowers ROS levels after treatment of astrocytes with H_2O_2 (100 μM). Left: representative photomicrographs of astrocytes stained with carboxy-H_2DCFDA to detect ROS and DAPI to stain cell nuclei. The increase in fluorescent cells elicited by H_2O_2 was markedly diminished by IGF-I. Right histograms: fluorimetric quantification of ROS levels with carboxy-H_2DCFDA confirmed the rescuing action of IGF-I on astrocytes exposed to H_2O_2. (F=7.362; **p<0.01 H_2O_2 vs. control, IGF-I or IGF-I + H_2O_2.
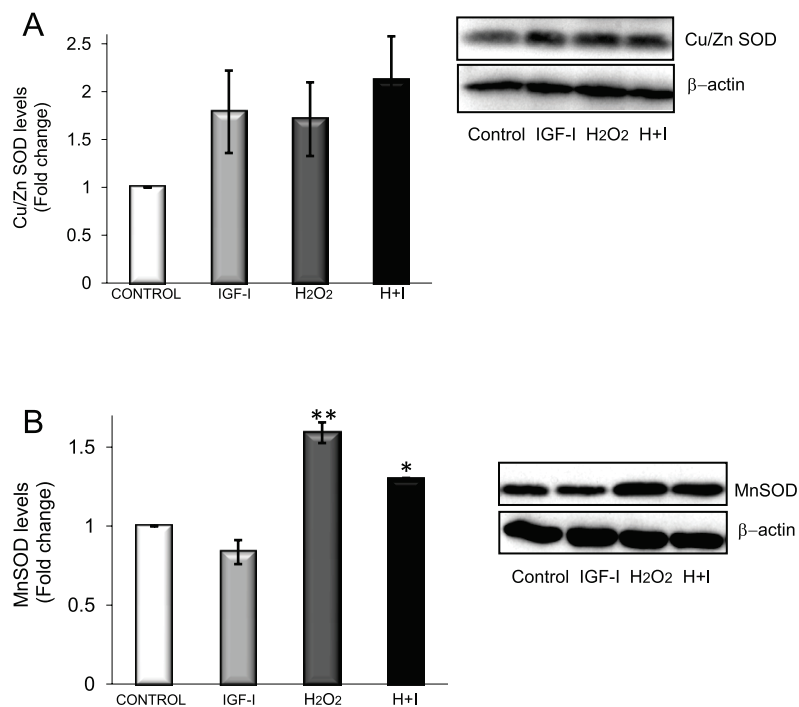

Figure 5. SOD responses to oxidative stress in astrocytes. A) Cu/ZnSOD levels in astrocytes are modulated by IGF-I and H_2O_2. **B**) MnSOD levels are enhanced by H_2O_2 but not by IGF-I (*p<0.05 and **p<0.01 vs. control).

to anti-oxidant responses[20]. We first confirmed that in astrocytes TXNIP1 is also controlled by FOXO; i.e.: in astrocytes expressing dominant negative Foxo, TXNIP1 levels were 89% reduced as compared to mock-transfected astrocytes. Accordingly, IGF-I, which inhibits FOXO, also reduced TXNIP1 levels (Figure 6A). Strikingly, H_2O_2, which stimulates FOXO activity in astrocytes (Figure 3D), also inhibited TXNIP1 (Figure 6A), suggesting alternative routes of TXNIP1 regulation in the presence of H_2O_2. When IGF-I and H_2O_2 were simultaneously added to astrocytes, TXNIP1 levels were markedly decreased ($p<0.05$ vs. IGF-I or H_2O_2 alone, Figure 6A). To determine the impact of downregulation of TXNIP1 on astrocyte survival we inhibited its expression with shRNA (blot in Figure 6B) and found that astrocytes became resistant to H_2O_2 when TXNIP1 levels were low (Figure 6B). Overexpression of TXNIP1 did not

alter the response of astrocytes to H_2O_2 whereas co-culture of neurons with astrocytes depleted of TXNIP1 did not result in enhanced neuronal survival (data not visualized for clarity), indicating that this route is involved in the response of astrocytes to oxidative stress but not in neuroprotection. Interestingly, in neurons, TXNIP1 was downregulated only in the presence of H_2O_2, but not after IGF-I treatment (Figure 6C). Thus, IGF-I downregulates TXNIP1 only in astrocytes, not in neurons.

We then analyzed possible pathways involved in the inhibitory effect of H_2O_2 and IGF-I on TXNIP1. Using kinase inhibitors we ruled out the idea that the main kinases downstream of the IGF-I receptor or H_2O_2 were involved. In fact, inhibition of most of these kinases resulted in altered basal levels of TXNIP1 (not visualized),
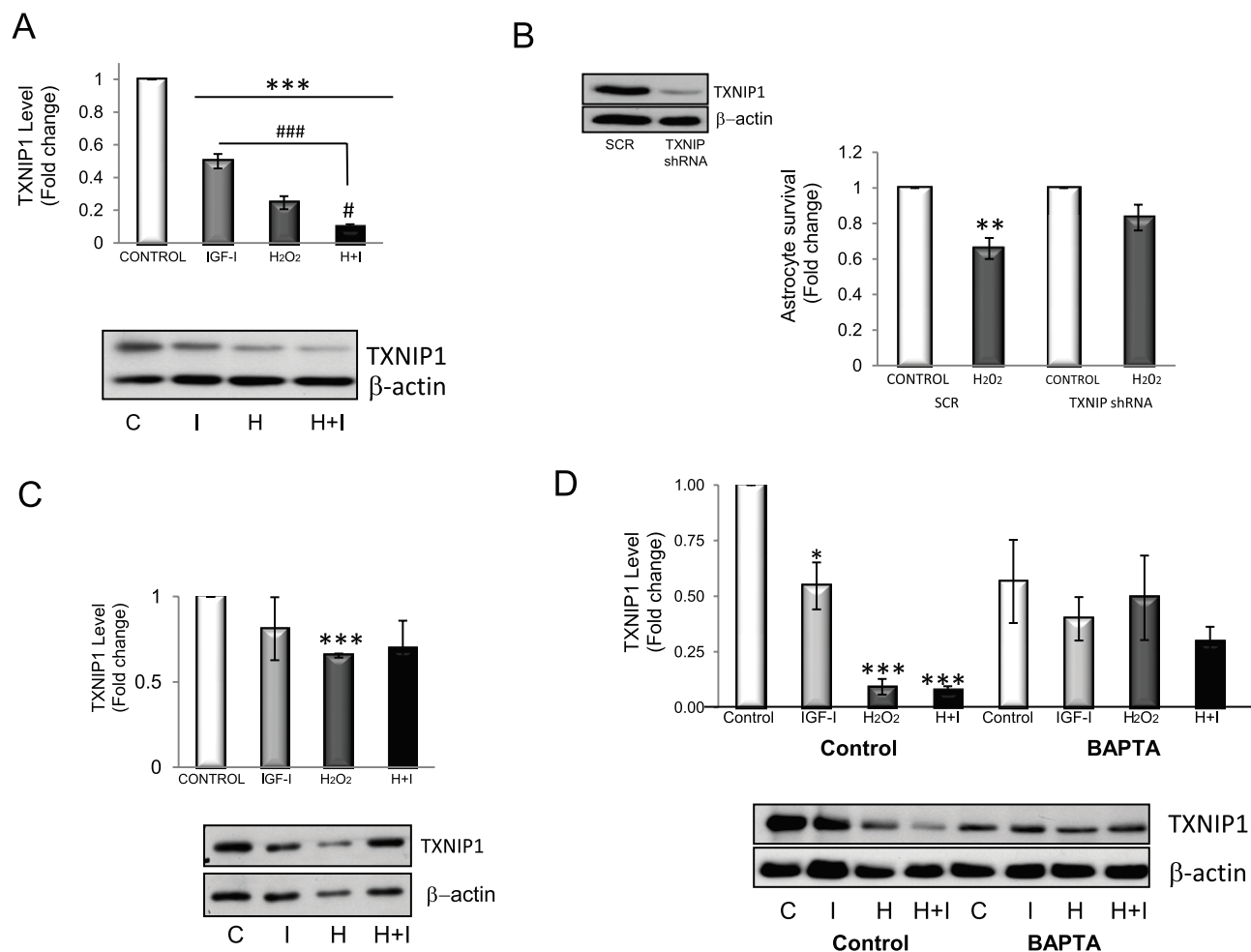

Figure 6. Both H_2O_2 and IGF-I reduce TXNIP1 in astrocytes. A) Levels of the pro-oxidant protein TXNIP1 are reduced by IGF-I and H_2O_2. Inhibition is greater when both are added together (F=156.6; ***$p<0.001$ vs. control and ###$p<0.001$ (vs. IGF-I) and #$p<0.05$ (vs. H_2O_2)). Levels of actin in each sample were measured to normalize TXNIP1 levels. **B**) Western blot: transfection of astrocytes with shRNA TXNIP1 results in reduced TXNIP1 levels as compared to astrocytes transfected with scrambled shRNA (SCR). TXNIP1 shRNA silencing makes astrocytes less sensitive to H_2O_2 toxicity. Astrocyte viability was measured by FDA in the presence of 200 µM H_2O_2 (F=12.09; **$p<0.01$ vs. control). **C**) In neurons, only H_2O_2 decreases TXNIP1 levels, whereas IGF-I does not (***$p<0.001$ vs. control). **D**) Reduction of TXNIP1 by IGF-I and H_2O_2 in astrocytes depends on Ca^{2+} as in the presence of the calcium chelator BAPTA-AM the decrease is abrogated, (F=7.226; *$p<0.05$ and ***$p<0.001$ vs. control). C=control, I=IGF-I, H=H_2O_2, H + I=H_2O_2 + IGF-I.

suggesting that basal levels of this protein are tightly regulated in astrocytes. Other inhibitory drugs of different pathways where IGF-I participates (PKC, PKA, CnA, PDK-1, NFκB among others) gave similar negative results. However, inhibition of Ca^{++} flux with 5 μM BAPTA abrogated TXNIP1 decreases in response to either H_2O_2 or IGF-I while only slightly, but not significantly affecting basal levels (Figure 6D).

IGF-I cooperates with SCF produced by astrocytes to protect neurons against oxidative stress

We next analyzed possible neuroprotective effects of IGF-I through astrocytes. Using a commercial gene array for growth factors we screened growth factor production by IGF-I-treated astrocytes in response to H_2O_2. Among the several growth factors that increased, stem cell factor (SCF) showed the highest elevation (Table 3). We confirmed by qPCR that SCF is increased after H_2O_2 whereas IGF-I decreased it (Figure 7A). As SCF has been shown to be neuroprotective[21], we determined whether it protects neurons against H_2O_2 and found that while SCF alone did not exert any protection, co-treatment with IGF-I resulted in significantly greater neuronal survival (p<0.05; Figure 7B). We then examined pathways underlying this cooperative action of IGF-I and SCF. Under basal conditions, the activity of extracellular signal-regulated kinase (Erk; measured as pErk/Erk ratio), a canonical kinase in IGF-I signalling, was increased by IGF-I as expected, and also by SCF (Figure 7C). Basal Erk activity was also increased by H_2O_2. However, Erk was no longer activated by IGF-I or SCF in the presence of H_2O_2. Only when both were added together to H_2O_2-challenged cultures Erk activity was increased (Figure 7C). No interactions were found with Akt, the other canonical kinase pathway activated by IGF-I.

To determine the *in vivo* relevance of these observations we submitted mice to brain ischemia as this brain insult is associated to oxidative stress[22], and both IGF-I[9] and SCF[23] have been shown to be neuroprotective after ischemia. We found that IGF-I mRNA is increased after middle cerebral artery occlusion (MCAO) both in the ipsilateral and contralateral cortex, while only the contralateral side showed increased SCF mRNA levels compared to intact mice (Figure 7D). However, levels of SCF protein were elevated after MCAO in both the damaged and contralateral sides compared to normal mice (Figure 7E). This suggests that after brain ischemia the contralateral cortex produces higher amounts of SCF that eventually reach the ischemic side. Under this condition IGF-I may interact with SCF to promote neuronal survival in the ipsilateral cortex.

Table 3. Growth factors array

Upregulated genes		Downregulated genes	
Gene symbol	**Fold regulation**	**Gene symbol**	**Fold regulation**
Bmp4	34.1544	Amh	**-43.2611**
Bmp8a	19.7667	Bdnf	**-5.4642**
IGF-I	**185.7219**	Bmp1	-51.304
IL7	**54.0417**	Bmp2	-8.3513
Inhbb	**595.9304**	Bmp3	**-311.6969**
SCF	**3072.0799**	Bmp6	-30.211
VEGFb	22.0239	Bmp7	**-40.3361**
		Clcf1	-7.5162
		Csf1	-6.4666
		Csf3	**-102.9643**
		Cxcl1	-35.4079
		Cxcl12	**-49.3166**
		Egf	-24.916
		Ereg	-10.8078
		Fgf1	-11.353
		Fgf10	-15.6273
		Fgf14	-12.7639
		Fgf18	-21.0245
		Fgf2	-9.6934
		Fgf22	-13.5011

A battery of growth factors was screened with an RT^2 Profiler™ PCR Array. In brief, astrocytes were treated or not with IGF-I+H_2O_2 for 16h and total RNA was isolated. After performing the RT-PCR, total cDNA was assayed for PCR Array. PCR data was analyzed with the RT^2 Profiler PCR Array Data Analysis version 3.5 software provided by the manufacturer. Significantly up- or downregulated genes are shown.
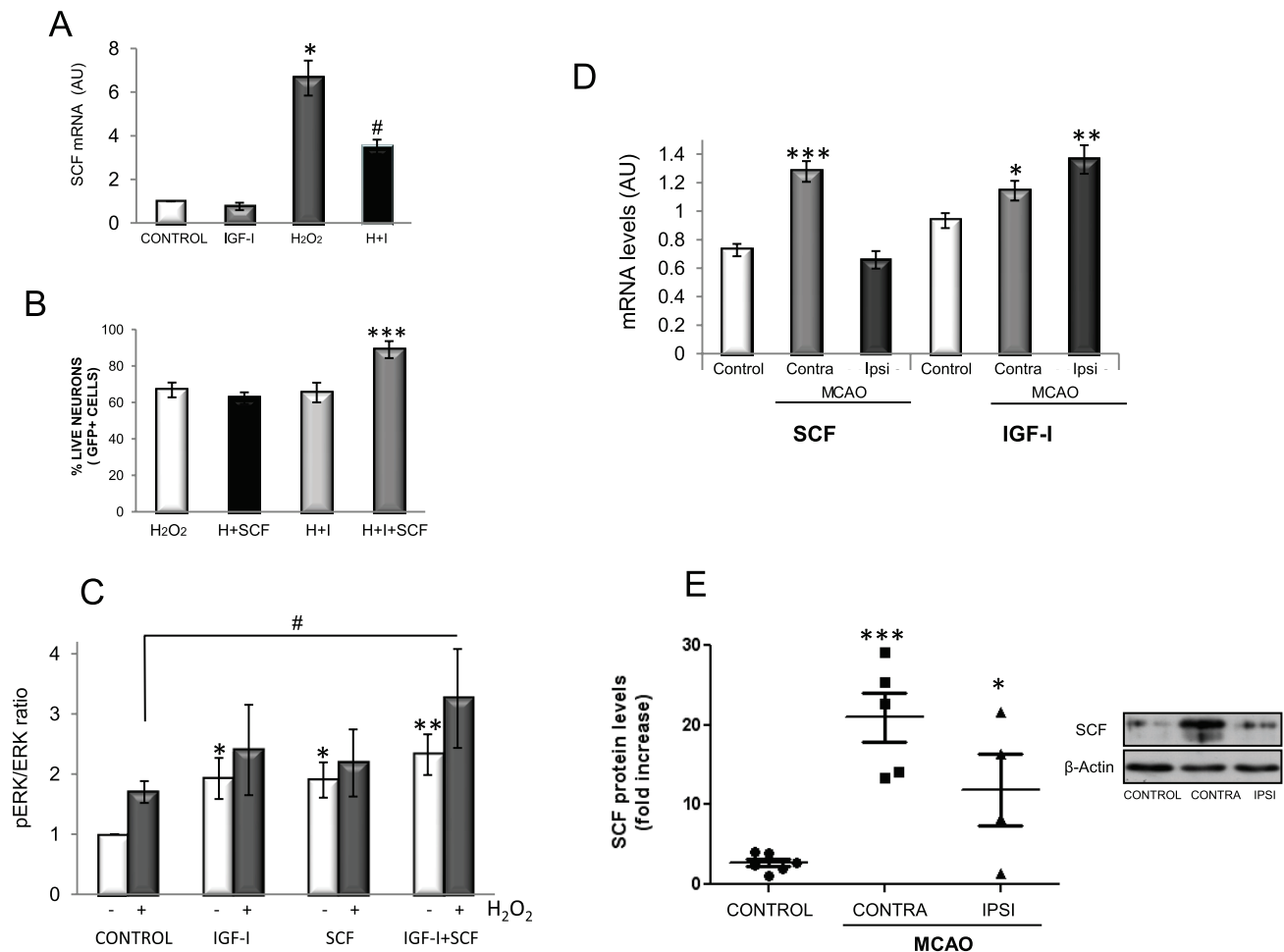
Figure 7. IGF-I cooperates with SCF to promote neuronal survival. A) H_2O_2 stimulates SCF mRNA levels in astrocytes after 16h of exposure whereas IGF-I partially counteracts this increase (F=38.67; *p<0.05 vs. control and IGF-I, #p<0.05 vs. H_2O_2). **B**) SCF and IGF-I cooperate to protect neurons from oxidative stress. Neurons were pretreated with SCF, IGF-I or both 48h before adding H_2O_2 (50 µM) and viability was assessed after overnight treatment (F=12.09, ***p<0.0001 vs. H_2O_2), H: H_2O_2; I: IGF-I. **C**) When H_2O_2 is present, Erk phosphorylation is significantly increased only when both SCF and IGF-I are added to the cultures but not with either alone. Neurons were treated with 100 nM IGF-I, 20 ng/ml SCF and 50 µM H_2O_2 for 5 minutes and pErk levels were measured by western blot and normalized for total Erk. (*p<0.05 and **p<0.01 vs. control without H_2O_2 and #p<0.05 vs. H_2O_2). **D**) SCF and IGF-I mRNA levels increased 16 hours after middle cerebral artery occlusion (MCAO) in the contralateral side (CONTRA) in the case of SCF (F=31.53; ***p<0.001 vs. intact control mice) and in both sides in the case of IGF-I (F=7.853; *p<0.05 and **p<0.01 vs. control). **E**) SCF protein levels increase after MCAO in both sides of the cortex. (F=12.38; *p<0.05 and ***p<0.001 vs. control). A representative blot is shown. Six, five and four animals were used per group, respectively. Levels of actin in each sample were measured to normalize for total protein levels.

Discussion

The present results indicate that IGF-I exerts a protective action on astrocytes contributing to the resilience of these glial cells against oxidative stress. IGF-I also cooperates with astrocytes to protect neurons. These observations highlight the importance of cell-specific and cell-cooperative aspects of IGF-I protection against oxidative challenge. Thus, a better understanding of the trophic role of IGF-I in the brain requires taking into account its effects on astrocytes (and other brain cells) and the functional links of these cells with neurons. While these observations do not help settle the role of oxidative stress in brain aging they put forward an important aspect of possible mechanisms involved in aging; regulatory signals such as IGF-I may not modulate the response of the different cells and even tissues to oxidative stress in the same way.

A greater resilience of astrocytes to oxidative stress provided by IGF-I will allow these cells to protect neurons that are more sensitive to oxidative challenge. Astrocytes are coupled to neurons in the response to oxidative stress and provide them with ample detoxification support[11]. In addition, among different anti-oxidant defences provided by astrocytes to neurons, we now find that IGF-I, which cannot protect isolated neurons against excess ROS[12] cooperates with SCF secreted by astrocytes to support neurons (Figure 8).
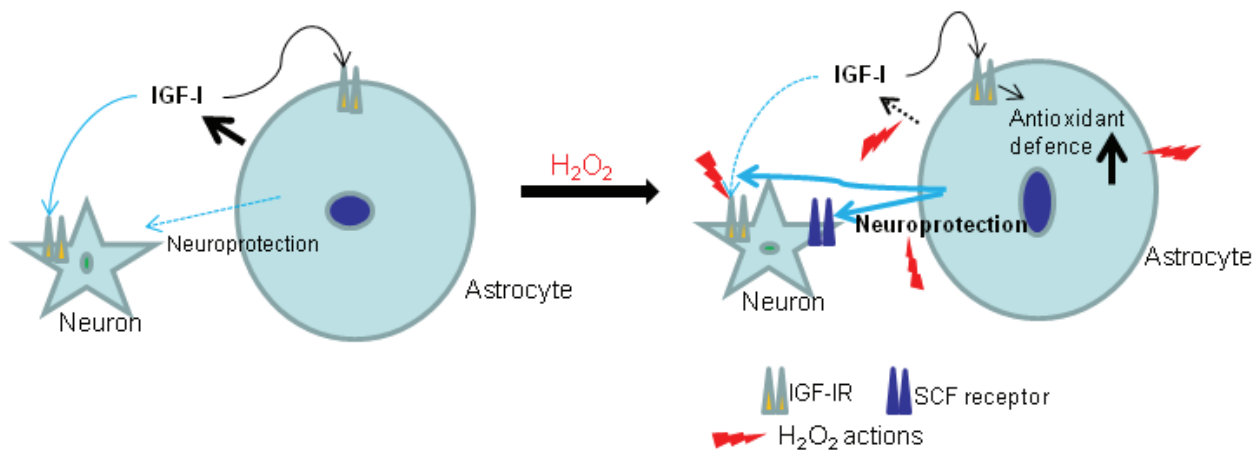
Figure 8. Schematic representation of IGF-I neuroprotection through astrocytes. Left: under basal conditions IGF-I exerts potent neuroprotective actions directly onto neurons and probably also through astrocytes. In the presence of H_2O_2 (right side), a number of events develop: 1) IGF-I loses its ability to directly protect neurons, 2) IGF-I secretion by astrocytes is diminished, 3) astrocytes reinforce anti-oxidant defences through IGF-I by downregulating pro-oxidant mechanisms such as TXNIP1, and 4) astrocytes potentiate neuroprotection by, among several other mediators, growth factors such as SCF that cooperate with IGF-I to protect neurons. Cytotoxic effects are depicted in red while cytoprotective actions are indicated in blue trace.

While in response to oxidative stress the production of IGF-I by cultured astrocytes and neurons is decreased, after brain ischemia IGF-I levels are actually higher due to increased synthesis and accumulation in microglia, vessels and astrocytes[24]. Therefore, *in vivo*, astrocytes and neurons will receive IGF-I input from various local sources, suggesting that the response of increased IGF-I after brain ischemia reflects an endogenous neuroprotective mechanism against oxidative injury. This conclusion apparently contradicts other evidence that IIS activity is pro-oxidant. Thus genetic ablation of ISS components in the nematode *Caenorhabditis elegans*[25] or in higher organisms such as the fruit fly[26] or mice[27], increases organism resistance to oxidative stress. For example, mice with reduced IGF-I activity (hemizygous for the IGF-I receptor) have lower levels of ROS in the brain[28,29]. However, these mice developed greater cell damage after oxidative injury[29]. Conceivably, the effects of modulating IGF-I signalling prior to ROS insult (as when using genetic models) may not be the same as after insult. For example IGF-I protects nerve cells and/or the brain against diverse types of ROS-related insults[30–34]. In this regard, we recently reported that in a cellular model of Friedreich's ataxia (which elicits oxidative damage) neurons responded to IGF-I only when they became frataxin deficient, but not under normal conditions[15]. Collectively these observations emphasize the importance not only of cell type but also of context dependency of IGF-I neuroprotection in relation to oxidative stress.

A role for oxidative stress in many neurodegenerative diseases is gaining increasing acceptance[35]. Aberrant production of ROS in the central nervous system is linked to neurodegenerative diseases such as Alzheimer´s dementia, Parkinson's disease or stroke, all of them associated to aging[36]. However, as already commented, the role of oxidative stress in brain aging is still unclear. An attempt to explain these apparently opposing observations is that moderate ROS levels may activate survival pathways[37]. The present findings agree with this proposal. Thus, doses of H_2O_2 up to 100 µM do not elicit astrocyte death probably because IGF-I helps maintain their anti-oxidant capacity and at the same time their neuroprotective action. In this regard our results show that astrocytes in response to IGF-I and/or H_2O_2 activate antioxidant signalling including upregulation of Cu/ZnSOD and MnSOD coupled to downregulation of pro-oxidant proteins such as Txnip1. Txnip1 inhibits thioredoxin (Trx), a protein that reduces protein disulfides as well as H_2O_2. The Txnip-Trx axis plays an important role in different brain diseases in which oxidative stress is implicated[38].

There is ample evidence that different trophic factors, including SCF[39], contribute to reduce cell damage due to oxidative stress after brain stroke[40]. We have found that *in vitro* IGF-I and SCF exert a cooperative neuroprotective effect against oxidative stress, suggesting that they may exert a similar beneficial role *in vivo* as after brain stroke both factors are upregulated in the lesioned area. Indeed, a cooperative neuroprotective effect of SCF with insulin has been reported[41]. The intracellular mechanisms mediating cooperation between these two factors involve Erk, a kinase activated by IGF-I.

In summary, cell specific and cooperative actions of IGF-I in brain responses to oxidative challenge underscores the need to design therapeutic strategies that take into account all aspects of biological organization, leading, for example, to cell-specific targeting of anti-aging drugs.

Data availability

figshare: Data on the responses of neurons and astrocytes to oxidative injury in the presence of insulin-like growth factor I, http://dx.doi.org/10.6084/m9.figshare.904909[70]

Author contributions

Laura Genis: designed, analyzed and performed experiments and wrote parts of the manuscript.

David Dávila: designed, analyzed and performed experiments and wrote parts of the manuscript.

Silvia Fernandez: designed, analyzed and performed experiments.

Andrea Pozo-Rodrigálvarez: performed experiments.

Ricardo Martínez-Murillo: performed experiments and contributed materials.

Ignacio Torres-Aleman: design the study, analyze the data and wrote the paper.

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Data Source

Current Referee Status: [?] ☑ ☑

Referee Responses for Version 1

☑ **Vince C Russo**
Centre for Hormone Research, Murdoch Children's Research Institute, Parkville, VIC, Australia

Approved: 06 March 2014

Referee Report: 06 March 2014

This manuscript by Laura Genis and co-workers is very interesting and the data presented are of significant scientific value. These studies provide further understanding of the cellular and molecular mechanisms involved in neuronal damage and rescue following oxidative stress. Of particular interest are the protective effects of IGF-I on neuronal cells mediated via the astrocytes. To these, the synergistic/additive effects of SCF on IGF-I are novel and interesting.

I have only few minor suggestions - mainly to improve graphic illustrations:

1. In figure 1B, IGF-I ELISA, the conditioned media from astrocytes and neuronal cells is analysed for IGF-I levels but the values are express in ng/ug of protein, why?

2. In Figure 3E I asume that the graph shows the % phosphorylation for the IGF-I treatment? This should be properly label.

3. In Figure 4 a statistical significance is shown for the 'untreated', but it should shown for the IGF-I which actually prevents MitoO2. I have a similar comment for the graph below in figure 4B.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

☑ **Carlos Matute**
Department of Neuroscience, País Vasco University, Leioa, Spain

Approved: 07 February 2014

Referee Report: 07 February 2014

This is an excellent paper with data relevant to CNS protection against oxidative stress.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

[?]

Marta Margeta

Department of Pathology, University of California San Francisco , San Francisco, CA, USA

Approved with reservations: 06 February 2014

Referee Report: 06 February 2014

In this paper, the authors attempt to elucidate the role of IGF-I in the astrocyte-mediated protection of neurons against oxidative stress. While this is an important topic and the authors present a lot of interesting data, the paper is unfocused and the results do not fully support the conclusions. As such, I feel that the paper cannot be approved for indexing until substantively revised.

Major comments:

1. The paper presents three essentially independent sets of data and then tries to connect them into a single coherent story, without direct experimental evidence that it is appropriate to do so. Specifically, Figs. 1 and 2 present evidence that IGF-I plays an important role in the astrocyte-mediated neuroprotection, both at baseline conditions and under oxidative stress; this is the most interesting part of the paper (and the part that is most relevant to the paper's current title). Figs. 3-6 show data that elucidate some aspects of IGF-I effect on astrocytes, but do not establish the importance of these effects/mechanisms for IGF-I- and astrocyte-mediated neuroprotection. (Notably, the authors have actually established that one of these mechanisms, IGF-I-induced decrease in the expression of astrocytic TXNIP1, does not play a role in the astrocyte-mediated neuroprotection. Surprisingly, these key results are not shown despite the fact that an entire figure [Fig. 6] is devoted to the IGF-I modulation of TXNIP1).

   Finally, Fig. 7 shows that IGF-I and SCF applied together (but not separately) have a neuroprotective effect in the absence of astrocytes and that their expression is increased in an *in vivo* stroke model. However, the authors again fail to show that these observations are in any way relevant for the astrocyte-mediated neuroprotection shown in Figs. 1 and 2. To connect these currently unconnected experimental threads, the authors need to use their neuron-astrocyte co-culture system to establish the link between astrocyte-mediated neuroprotection and (1) IGF-I-mediated increase in the expression of astrocyte antioxidant enzymes, (2) IGF-I-mediated decrease in the astrocyte ROS levels, and (3) astrocyte secretion of SCF; the experiments should be performed both at baseline and under oxidative stress conditions. Alternatively, the paper needs to be re-written in a way that makes it very clear (1) that the data presented in the paper represent a series of independent observations that do not add up to a coherent whole and (2) that the mechanism mediating IGF-I-induced neuroprotection in the mixed neuron-astrocyte environment currently remains unexplained. If choosing this route, the authors should also change the title of the paper to something more neutral and descriptive.

2. The authors do not show some important experimental data, ostensibly "for clarity"; these data need to be included in the revised paper. Specifically, as already stated in point #1, the authors mention (but do not show) that depleting astrocytes of TXNIP1 does not result in increased neuronal survival (page 16). This finding, if properly established, indicates that the IGF-I-mediated decrease in TXNIP1 and the IGF-I/astrocyte-mediated neuroprotection are two entirely unrelated phenomena. Given the paper's overall title and conclusions, this is a key experiment that needs to be shown. Similarly, it is important to show the results of IFG-IR DN experiment performed under oxidative stress conditions (page 7).

3. In many experiments, the authors do not use appropriate statistical analyses. Specifically, given the experimental design, two-way (rather than one-way) ANOVA should be used to analyze data shown in Figs. 1B-C, 1D (after missing data are included), 2A-E, 3A-D, 4A-B, and 6B.

4. Fig. 8 (the model) does not accurately represent the experimental results. For example, the authors state in the Fig. 8 legend that "*under basal conditions IGF-I exerts potent neuroprotective actions directly onto neurons*" when in fact, IGF-I has no clear neuroprotective effect when applied to neurons cultured alone (Fig. 3A, 0 μM H_2O_2 condition) – the IGF-I-induced decrease in neuronal cell death is small [~5% based on the graph] and does not appear statistically significant, although the authors do not comment on this one way or the other. Similarly, the figure legend mentions that IGF-1 down-regulates astrocytic TXNIP1 – a finding that is accurate but not relevant for the astrocyte-mediated neuroprotection illustrated by the figure. Thus, Fig. 8 should either be altered to more meaningfully represent the paper's findings or, if the authors decide to re-write the paper in a more descriptive fashion, could be eliminated altogether.

Minor comments:

1. Why are neurons cultured under depolarizing conditions (25 mM KCl)?

2. The co-culture experimental set-up should be described under a separate heading, not buried under "**Cell assays**".

3. For similar experiments, the authors should use a similar type of plot to make the paper more readable. For example, experiments in Figs. 3A and 3B have a very similar overall design – why are the results shown very differently?

4. Neuronal viability is established by counting "*all GFP-positive cells*"; the authors therefore need to show that their neuronal cultures are at least 95% and preferably 99% pure (i.e., do not contain a significant population of GFP-positive glial cells).

5. Representative flow cytometry plots should be included in Fig. 4A.

6. To enhance readability, all figure panels should be labelled "astrocytes", "neurons", or "neurons + astrocytes", as appropriate. (The information is currently largely buried in the figure legends.)

7. In Fig. 5, authors should specify whether they are measuring mRNA or protein level (I assume the latter, but it's difficult to be sure).

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Article Comments

Comments for Version 1

Author Response

Ignacio Torres Aleman, Cajal Institute, Spain
Posted: 06 Feb 2014

We appreciate the comments of the reviewer. We understand that we did not succeed in conveying the notion that IGF-I exerts specific actions on astrocytes related to Txnip1 ...etc that do not relate directly to neuroprotection by astrocytes; we may call it "*astroprotection by IGF-I*". We will carefully re-write the manuscript to deal with this problem. The experiments with SCF were already done but not included in the manuscript so we now can incorporate them: SCF secretion by astrocytes increases in response to oxidative stress. We´ll introduce the required changes once we received the assessment of the rest of the referees. We thank the reviewer for her careful insight into our work that will substantially improve it.

Competing Interests: No competing interests were disclosed.