γ-Glutamylcysteine detoxifies reactive oxygen species by acting as glutathione peroxidase-1 cofactor

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Reactive oxygen species regulate redox-signaling processes, but in excess they can cause cell damage, hence underlying the aetiology of several neurological diseases. Through its ability to down modulate reactive oxygen species, glutathione is considered an essential thiol-antioxidant derivative, yet under certain circumstances it is dispensable for cell growth and redox control. Here we show, by directing the biosynthesis of γ-glutamylcysteine—the immediate glutathione precursor—to mitochondria, that it efficiently detoxifies hydrogen peroxide and superoxide anion, regardless of cellular glutathione concentrations. Knocking down glutathione peroxidase-1 drastically increases superoxide anion in cells synthesizing mitochondrial γ-glutamylcysteine. In vitro, γ-glutamylcysteine is as efficient as glutathione in disposing of hydrogen peroxide by glutathione peroxidase-1. In primary neurons, endogenously synthesized γ-glutamylcysteine fully prevents apoptotic death in several neurotoxic paradigms and, in an in vivo mouse model of neurodegeneration, γ-glutamylcysteine protects against neuronal loss and motor impairment. Thus, γ-glutamylcysteine takes over the antioxidant and neuroprotective functions of glutathione by acting as glutathione peroxidase-1 cofactor.

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mitochondria are both producers and targets of reactive oxygen species (ROS). A well-balanced equilibrium between mitochondrial ROS production and their elimination is critical for the control of redox-mediated cell signalling processes. By disposing ROS, glutathione (γ-glutamylcysteinylglycine, GSH) is thought to be the main small thiol-antioxidant derivative. GSH is synthesized exclusively in the cytosol in two consecutive ATP-requiring steps, the first of which, and rate limiting, being γ-glutamylcysteine formation by glutamate-cysteine ligase (GCL); glutathione synthetase (GSS) then forms the tripeptide by linking glycine to γ-glutamylcysteine.

It is well documented that pharmacological inhibition of GCL activity or knockdown of the catalytic GCL subunit increases ROS abundance, which mediates cellular damage; GCL genetic deletion is embryonically lethal.

Interestingly, GSH is dispensable for the cell growth of Saccharomyces cerevisiae genetically deleted of GSS. In addition, it has been recently demonstrated that, in S. cerevisiae devoid of GSS, GSH is essential for iron–sulfur cluster assembly, but not for thiol-redox control. Both in yeast and in human fibroblasts, genetic GSS deletion results in γ-glutathione accumulation; thus the antioxidant function of GSH might be adopted by γ-glutamylcysteine. Here we tested whether—and if so, how—γ-glutamylcysteine detoxifies hydrogen peroxide (H2O2) and superoxide anion (O2−) under intact, physiological GSH concentrations. To do so, GCL was directed to mitochondria, as this organelle cannot further transform γ-glutamylcysteine into GSH due to the absence of GSS. We found that both mitochondrial-targeted newly synthesized, and endogenous γ-glutamylcysteine, efficiently dispose H2O2 by acting as glutathione peroxidase-1 cofactor. These results indicate that γ-glutamylcysteine is an important player in cellular redox control.

**Results**

Targeting functional GCL to mitochondria decreases ROS. To direct γ-glutamylcysteine synthesis to mitochondria, the full-length complementary DNA encoding the catalytic GCL subunit was fused with the mitochondrial-targeting signal of ornithine transcarbamylase. When expressed in HEK293T cells, this construct (mitoGCL) yielded a GCL protein that was confined to mitochondria and absent in the cytosol, as revealed by western blotting after cellular fractionation (Fig. 1a) and confocal microscopy (Fig. 1b). In contrast, expression of untagged GCL was exclusively present in the cytosol (Fig. 1a,b). Whole-cell extracts expressing mitoGCL revealed a super-shifted anti-GCL (1:1,000) band that was not present in the untagged GCL-expressing cells or in the mitoGCL-transfected mitochondria (Fig. 1c), suggesting that only the mitochondrial-targeting epitope–processed form of the protein was present in the mitochondrial fraction.

Then, we assessed whether expressed mitoGCL yielded functional GCL within mitochondria. As shown in Fig. 1d (left panel), GCL activity was undetectable in mitochondria isolated from control cells, but present in mitoGCL-transfected cells. Furthermore, neither mitochondrial (Fig. 1d, middle panel) nor cytosolic (Supplementary Fig. S1a) GSH concentrations changed by mitoGCL expression. Interestingly, H2O2 detection was significantly diminished by mitoGCL expression, as measured both in mitochondria (Fig. 1d, right panel) and in intact cells (Supplementary Fig. S1b,c). Expression of mitoGCL prevented the conversion of GSH to its oxidized form (GSSG) caused by rotenone (Supplementary Fig. S1d). To confirm that GCL activity wholly accounted for ROS down-modulation, we expressed (Fig. 1e) a E103A mutant inactive (Fig. 1f, left panel) form of mitoGCL. We found that rotenone-induced mitochondrial O2− was significantly decreased by wild-type mitoGCL, but not by the E103A inactive form (Fig. 1f, right panel). These data confirm that γ-glutamylcysteine is required for the observed antioxidant function.

γ-Glutamylcysteine is a GPx1 cofactor that detoxifies H2O2. To decipher how γ-glutamylcysteine detoxified ROS but otherwise avoiding the influence of cytosolic GCL, we expressed a silent mutant mitoGCL form (mitoGCL(mut)) refractory to the action of a small hairpin RNA against GCL (shGCL), in HEK293T cells (Fig. 2a). Knocking down GCL markedly reduced total cellular GSH in both mitoGCL and mitoGCL(mut) transfected cells (Supplementary Fig. S1e). GCL knockdown was insufficient to trigger a significant increase in mitochondrial O2− but this was strongly potentiated by rotenone and rescued by mitoGCL (mut; Fig. 2b). Thus, in untransfected HEK293T cells, γ-glutamylcysteine may not contribute to the basal O2− regulation that can occur in primary neurons, in which we previously reported a significant increase in O2− by shGCL. It therefore appears that the impact of γ-glutamylcysteine as a physiological redox regulator differs among cell types.

Next, we knocked down GSS (Fig. 2c), which resulted in endogenous γ-glutamylcysteine accumulation and GSH decrease (Supplementary Table S1). We found that GSS knockdown abolished the increase in rotenone-induced O2−, both in the absence (Supplementary Fig. S1f) and in the presence of mitoGCL (Fig. 2d).

We then sought to elucidate if γ-glutamylcysteine served as a glutathione peroxidase cofactor. As glutathione peroxidase-1 (GPx1) largely accounts for mitochondrial H2O2 detoxification, we knocked down it (Fig. 2c), which resulted in a significant enhancement of rotenone-induced O2− (Fig. 2d). Furthermore, GSS knockdown was unable to decrease O2− levels during GPx1 silencing (Fig. 2d). The oxidized form of γ-glutamylcysteine was undetectable in cultured cells (Supplementary Table S1), but it was present in all tissues analysed in vivo (brain, liver and kidney; Supplementary Table S2). Knocking down glutathione reductase (GSR) failed to enhance rotenone-induced O2− (Fig. 2d). To disregard a direct interaction of γ-glutamylcysteine with O2−, we silenced the mitochondrial isoform of superoxide dismutase (SOD2; Fig. 2c), which enhanced rotenone-induced O2− (Fig. 2d). The ability of either γ-glutamylcysteine or its cognate cofactor, GSH, to dispose H2O2 in vitro was then assessed in the absence or presence of purified GPx1. As shown in Fig. 2e, γ-glutamylcysteine and GSH were unable to detoxify H2O2 unless GPx1 was present; γ-glutamylcysteine dose-dependently accelerated GPx1-mediated H2O2 disposal at a similar efficiency to that by GSH, at least at low concentrations of the thiols (Fig. 2e). GSR failed to improve the GPx1-dependent ability of γ-glutamylcysteine, but not that of GSH, at disposing H2O2 (Fig. 2f).

We also tested the ability of γ-glutamylcysteine to induce protein modification, which was found to be well below that of GSH (Supplementary Table S3).

**mitoGCL decreases ROS in neurons and is neuroprotective.** Neurons are particularly vulnerable against excess ROS, hence requiring continuous supply and regeneration of GSH for survival. We therefore investigated the possible efficacy of γ-glutamylcysteine at detoxifying ROS in (patho)physiologically relevant neuronal death models. Glutamate treatment increased mitochondrial O2− in rat primary neurons (Fig. 3a; Supplementary Fig. S2a), an effect that was abolished by the N-methyl-D-aspartate receptor antagonist, MK801 (Supplementary Fig. S2b). Expression of mitoGCL in neurons was sufficient to decrease basal mitochondrial O2− (Fig. 3a), which contrasts with the lack of effect in HEK293T cells (Fig. 1f).

The different response of these cells to mitoGCL expression is likely due to the above-mentioned vulnerability of neurons to oxidative stress versus the resistance of HEK293T cells (Fig. 2b). Furthermore, mitoGCL, but not its inactive E103A mutant form, prevented the increase in mitochondrial O2− induced by glutamate-receptor stimulation (Fig. 3a). GPX1—but not GSS or GSR—knockdown (Supplementary Fig. S2c) significantly enhanced O2− (Fig. 3b), despite neurons expressed mitoGCL, indicating that GPX1 use of γ-glutamylcysteine is essential for γ-glutamylcysteine-mediated O2− detoxification in this model of excitotoxicity. Glutamate triggered an increase in the proportion of neurons with active caspase-3 (Fig. 3c) and
with annexin V+7-AAD− staining (Fig. 3d), indicating an intrinsic (mitochondrial) mode of apoptotic death; this was prevented by antagonizing the N-methyl-D-aspartate receptors (Supplementary Fig. S2d,e). Notably, mitoGCL largely—but not fully—prevented the rise in the percentage of neurons with the apoptotic phenotype (Fig. 3c,d). In addition, mitoGCL abolished O2−−−− enhancement triggered by other mitochondrial ROS-inducing agents17,18, such as rotenone, antimycin and 3-nitropropionic acid (3NP; Fig. 3e).

**mitoGCL exerts neuroprotection in vivo.** To confirm that γ-glutamylcysteine exerted neuroprotection in vivo, lentiviral particles expressing wild-type or inactive (E103A) mitoGCL were stereotactically injected into the striatum of adult mice. After 3 days, striatal GCL activity was significantly higher in mice injected with mitoGCL than in those injected with inactive mitoGCL (E103A), whereas striatal GSH concentrations remained unchanged (Fig. 4a). After 3 days of lentiviral injections, mice were intraperitoneally injected with 3NP (seven doses of 50 mg·kg−1, twice daily), a well-described model of neurodegeneration18. Indeed, we observed that 3NP treatment induced a significant increase in neuronal apoptotic death in the striatum, as judged by TdT-mediated dUTP nick end labelling assay, in the mice pre-injected with inactive mitoGCL (E103A), but not in those pre-injected with wild-type mitoGCL (Fig. 4b,c). Notably, there was a significant loss of striatal neurons in the mice pre-injected with the inactive mitoGCL (E103A), but not in those pre-injected with the wild-type mitoGCL (Fig. 4d; Supplementary Fig. S2f). Mice treated with vehicle instead of 3NP showed no neuronal loss, regardless of the isoform of mitoGCL (wild type or inactive) pre-injected (Fig. 4d; Supplementary Fig. S2f). To evaluate behavioural function, these mice were tested for motor coordination and balance. As shown in Fig. 4e, 3NP treatment induced a progressive motor impairment in the mice that were pre-injected with the inactive mitoGCL (E103A), but not in those pre-injected with the wild-type mitoGCL; mice treated with vehicle instead of 3NP showed no motor impairment, regardless of the isoform of mitoGCL (wild type or inactive) pre-injected (Fig. 4e).

**Discussion** Here we show that γ-glutamylcysteine is a thiol-redox regulator that efficiently detoxifies mitochondrial ROS. This was evidenced
by confining γ-glutamylcysteine synthesis to mitochondria, which were unable to convert the newly synthesized γ-glutamylcysteine into GSH due to the lack of mitochondrial GSS\textsuperscript{13}. Thus, down-modulation of mitochondrial ROS by γ-glutamylcysteine took place under physiological GSH concentrations. Furthermore, this robust antioxidant protection observed in intact GSS knockdown cells corroborates such a role by endogenous γ-glutamylcysteine. Notably, impairing γ-glutamylcysteine biosynthesis in the cytosol, but not in mitochondria, still allowed γ-glutamylcysteine to efficiently detoxify ROS. Together, these results indicate that the antioxidant action of γ-glutamylcysteine takes place regardless of GSH concentrations, thus cannot simply rely on its ability to replenish GSH, as it can be seen in other paradigms\textsuperscript{19}.

Our results also reveal that GPx1 is an essential component in the detoxifying function of γ-glutamylcysteine. Furthermore, the occurrence of an oxidized form of γ-glutamylcysteine in all tissues analysed, in vivo, strongly suggests the notion of a γ-glutamylcysteine redox cycle. However, knocking down GSR—presumably required for an eventual regeneration of reduced γ-glutamylcysteine from its oxidized form—failed to enhance O\textsubscript{2}⁻\textsuperscript{−}. The biochemical pathway responsible for the reduction of γ-glutamylcysteine from its oxidized form therefore remains to be elucidated. On the other hand, a putative function of γ-glutamylcysteine as direct superoxide anion scavenger is disregarded, as SOD2 knockdown could not further enhance rotenone-induced O\textsubscript{2}⁻\textsuperscript{−}. Our data obtained by knocking down GPx1, both in the human HEK293T cells and in rat neurons, together with the in vitro experiments, strongly suggest that endogenous γ-glutamylcysteine is a GPx1 cofactor for H\textsubscript{2}O\textsubscript{2} detoxification. It cannot be ruled out that γ-glutamylcysteine could also act as a cofactor for other isofoms of the glutathione peroxidase family; however, GPx1 largely accounts for most mitochondrial H\textsubscript{2}O\textsubscript{2} detoxification\textsuperscript{14}, suggesting a major role for this isoform. Moreover, previous studies have shown that GPx1 can accept, albeit at a much lower efficiency, a range of thiol-derivatives besides GSH as the electron donor\textsuperscript{20}. The essential cysteiny1-sulphydryl and glutamyl-carboxylic groups for GPx1 active site interaction and catalysis\textsuperscript{14} are both preserved in γ-glutamylcysteine, indicating the structural feasibility for this function.

Due to its very low ability to synthesize and regenerate GSH, the brain is one of the most vulnerable tissues to excess ROS\textsuperscript{15,21}. In Parkinson’s disease, there is an ~60% reduction in GSH concentration in the substantia nigra of presymptomatic patients\textsuperscript{22}, suggesting GSH deficiency as one of the earliest biochemical signs of this disorder. Moreover, signs of excess ROS are associated with
the pathophysiology of other neurodegenerative diseases, such as Alzheimer’s, or disorders affecting motor functional disturbances such as Amyotrophic Lateral Sclerosis or Huntington’s disease. At the light of our data, GPx1 is essential for γ-glutamylcysteine-mediated ROS detoxification and neuroprotection against different insults triggering neurodegeneration in primary neurons. Furthermore, we also show neuroprotection and motor improvement in an in vivo mouse model of neurodegeneration. Thus, targeting γ-glutamylcysteine to neuronal mitochondria represents an improvement over the protection activity of neighbouring astrocytes.

In conclusion, our results demonstrate that γ-glutamylcysteine is a thiol-redox regulator that efficiently detoxifies mitochondrial ROS through GPx1. In the presence of γ-glutamylcysteine taking an antioxidant function, GSH stops from being oxidized; moreover, the ability of γ-glutamylcysteine to induce protein modification is well below that of GSH. Thus, when γ-glutamylcysteine takes the antioxidant functions, GSH utilization may be preserved for iron–sulfur cluster assembly and protein thiol-redox modifications, as recently reported. How the antioxidant role of γ-glutamylcysteine is regulated under (patho) physiological conditions now needs to be deciphered. In this context, it should be noted that, besides GCL, alternative pathways can account for intracellular γ-glutamylcysteine. For instance, γ-glutamyltransferase forms γ-glutamylcysteine at the extracellular side of the plasma membrane and then is taken up by the cells through the glutamyl-amino acid transporter. It would be interesting to investigate if increased γ-glutamylcysteine could explain the drug resistance observed in tumours associated with γ-glutamyltransferase overexpression. In addition, whether GSH deficiency is the prime mode of the pro-oxidant actions of the GCL/GSS/glutamyl-amino acid transporter inhibitor, l-buthionine sulfoximine needs to be revisited. Finally, our results, showing neuroprotection by mitochondrial-targeted γ-glutamylcysteine biosynthesis, support the oxidative hypothesis of neurodegeneration. Although classical antioxidant drugs failed in clinical trials against neurodegenerative disorders, mitoGCL, by acting as a persistent antioxidant function, GSH utilization may be preserved for iron–sulfur cluster assembly and protein thiol-redox modifications, as recently reported. How the antioxidant role of γ-glutamylcysteine is regulated under (patho) physiological conditions now needs to be deciphered. In this context, it should be noted that, besides GCL, alternative pathways can account for intracellular γ-glutamylcysteine. For instance, γ-glutamyltransferase forms γ-glutamylcysteine at the extracellular side of the plasma membrane and then is taken up by the cells through the glutamyl-amino acid transporter. It would be interesting to investigate if increased γ-glutamylcysteine could explain the drug resistance observed in tumours associated with γ-glutamyltransferase overexpression. In addition, whether GSH deficiency is the prime mode of the pro-oxidant actions of the GCL/GSS/glutamyl-amino acid transporter inhibitor, l-buthionine sulfoximine needs to be revisited. Finally, our results, showing neuroprotection by mitochondrial-targeted γ-glutamylcysteine biosynthesis, support the oxidative hypothesis of neurodegeneration. Although classical antioxidant drugs failed in clinical trials against neurodegenerative disorders, mitoGCL, by acting as a persistent...
were generated following a standard protocol to generate blunt ends, and then subcloned in the mitochondria of rat (mito) Inc., Palo Alto, CA, USA) with GATTAAACAGCATA-3′ GAAGTTGTGACCATTTCTAAAAGCTGCATTGTTTAACAGGATCCTCA and 5′-CACAACTTCATGGTTCGAAATTTTCGGTGTGGACAACCACTACAAG-3′ transcarbamylase (GenBank accession number NM_000531). The forward and reverse 64-nt-long oligonucleotides were 5′-AGAAGTTGTGACCATTTCTAAAAGCTGCATTGTTTAACAGGATCCTCA and 5′-CACAACTTCATGGTTCGAAATTTTCGGTGTGGACAACCACTACAAG-3′ with a cDNA fragment encoding the first 32 amino acids of human ornithine decarboxylase (ODC). ODC and ODC knockdown by short interfering RNA (siRNA). For GSS: 5′-GGACCTGAGTTTAAACAAA-3′ (nucleotides 338–356 for both human, NM_00581, and rat, NM_017051) and 5′-GGACCTGAGTTTAAACAAA-3′ (nucleotides 1154–1172 for rat NM_053906). For glutathione peroxidase-1 (GPx1): 5′-CGCCAAGAACGCTGAGTTTAAACAAA-3′ and 5′-CGCCAAGAACGCTGAGTTTAAACAAA-3′. The firefly luciferase-targeted oligonucleotide 5′-CTGACGCGGAATACTTCGA-3′ was used as control. The forward and reverse 64-nt-long oligonucleotides were annealed and inserted into the BglII/HindIII sites of pSuper-neo/GFP vector (OligoEngine, Seattle, WA). These constructions concomitantly express 19-bp, 9-nucleotide stem-loop shRNAs with GFP, thus allowing the identification of transfected cells by fluorescence microscopy and flow cytometry.

Small interfering RNA construction. Using previously reported rational criteria, we designed the following target sequences to get gene expression-specific knockdown by short interfering RNA (siRNA). For GSS: 5′-AGAAGTTGTGACCATTTCTAAAAGCTGCATTGTTTAACAGGATCCTCA and 5′-CACAACTTCATGGTTCGAAATTTTCGGTGTGGACAACCACTACAAG-3′. The firefly luciferase-targeted oligonucleotide 5′-CTGACGCGGAATACTTCGA-3′ was used as control. The forward and reverse 64-nt-long oligonucleotides were annealed and inserted into the BglII/HindIII sites of pSuper-neo/GFP vector (OligoEngine, Seattle, WA). These constructions concomitantly express 19-bp, 9-nucleotide stem-loop shRNAs with GFP, thus allowing the identification of transfected cells by fluorescence microscopy and flow cytometry.

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a siRNA against luciferase (5′-CTGACGCGAGAATCTCGAGA-3′) was used as control. siRNAs were purchased from Dharmacon (Abgene, Thermo Fisher, Epsom, UK).

**Site-directed mutagenesis.** Mutant forms of mitoGCL refractory to the siRNA against GCL (mitoGCLmut) or catalytically inactive mitoGCL (mitoGCL(E103A)) were generated using the site-directed mutagenesis QuikChange XL kit (Stratagene, La Jolla, CA, USA), followed by Q5 polymerase-directed digestion. The wild-type and reverse oligonucleotides of the sequence 5′-GAAAGAAGACAGCTGATG-3′, carrying silent third-codon base point mutations (mutant nucleotides underlined) were used for mitoGCLmut. To generate mitoGCL(E103A), the forward and reverse oligonucleotides 5′-CAGAATGTGGAAGATCTTGAGA-3′ (mutant nucleotides underlined) were used.

**Cell cultures.** Cortical neurons in primary culture were prepared from fetal Wistar rats (E16), seeded at 2.5×10^4 cells per cm² in 6- or 12-well plates previously coated with poly-D-lysine (15 µg ml⁻¹) in DMEM (Sigma, Madrid, Spain) supplemented with 10% and 1% of fetal bovine serum, respectively (Berlin, Germany). Cells were incubated at 37°C in a humidified 5% CO₂-containing atmosphere. At 48 hours after plating, the medium was replaced with DMEM supplemented with 5% horse serum (Serum, USA). Immunoblotting was performed with rabbit polyclonal anti-GFAP (1:1000) (Abcam), mouse monoclonal anti-βIII-tubulin (1:1000) (Abcam), mouse monoclonal anti-β-actin (1:1000) (Sigma, USA) fluorimeter (excitation: 538 nm, emission: 604 nm), and the slopes were used for calculations. For H₂O₂ quantification was assessed as described³⁶ by the BCA Protein Assay kit (Pierce), following the manufacturer’s instructions. In both cases, bovine serum albumin was used as standard.

**Primary antibodies for western blotting.** Immunoblotting was performed with rabbit polyclonal anti-GCL (1:1000), rabbit polyclonal anti-GPX1 (1:1000) (Abcam), rabbit polyclonal anti-GFP (1:1000) (Abcam), mouse monoclonal anti-β-actin (1:1000) (Sigma, USA), mouse monoclonal anti-βIII-tubulin (1:1000) (Abcam), mouse monoclonal anti-β-actin (1:1000) (Sigma, USA) fluorimeter (excitation: 538 nm, emission: 604 nm), and the slopes were used for calculations. For H₂O₂ quantification was assessed as described³⁶ by the BCA Protein Assay kit (Pierce), following the manufacturer’s instructions. In both cases, bovine serum albumin was used as standard. Statistical analysis of the results was performed by one-way analysis of variance, followed by the least significant difference multiple range test or by the Student’s t-test for comparisons between two groups of values. In all cases, P<0.05 was considered significant.

**Use of animals.** All animals used in this work were obtained from the Animal Experimentation Unit of the University of Salamanca, in accordance with Spanish legislation (RD 1201/2005) under licence from the Spanish Ministry of Science and Innovation. Protocols were approved by the Bioethics Committee of the University of Salamanca.

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### Author contributions

J.P.B. conceived the idea. J.P.B., R.Q.-C. and A.A. designed research. R.Q.-C., S.F.-F., V.B.-J., J.E., J.S. and A.A. performed research. J.P.B., R.Q.-C. and A.A. analysed the data. J.P.B. wrote the paper.

### Additional information

**Supplementary Information** accompanies this paper at [http://www.nature.com/naturecommunications](http://www.nature.com/naturecommunications).  

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