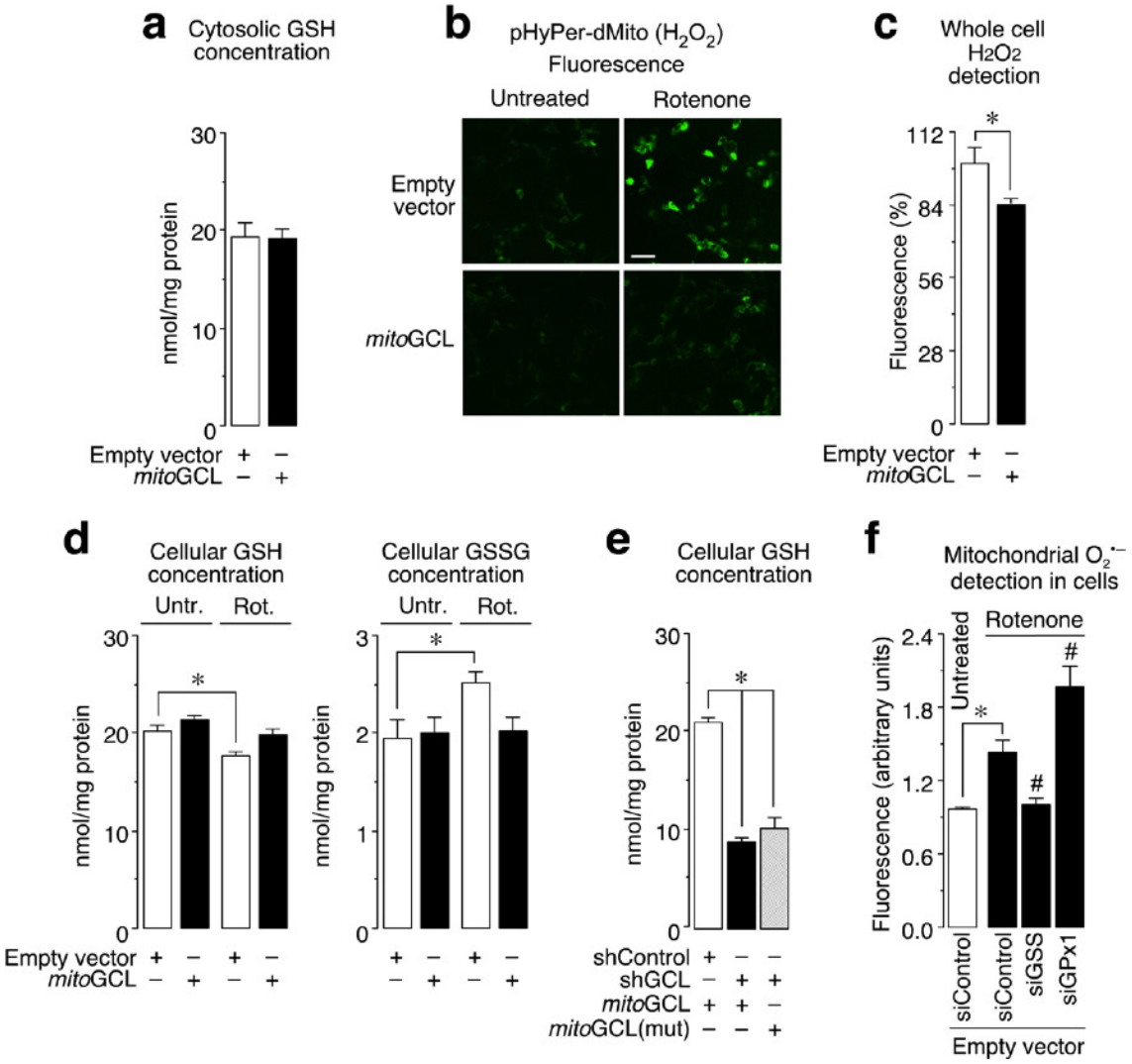
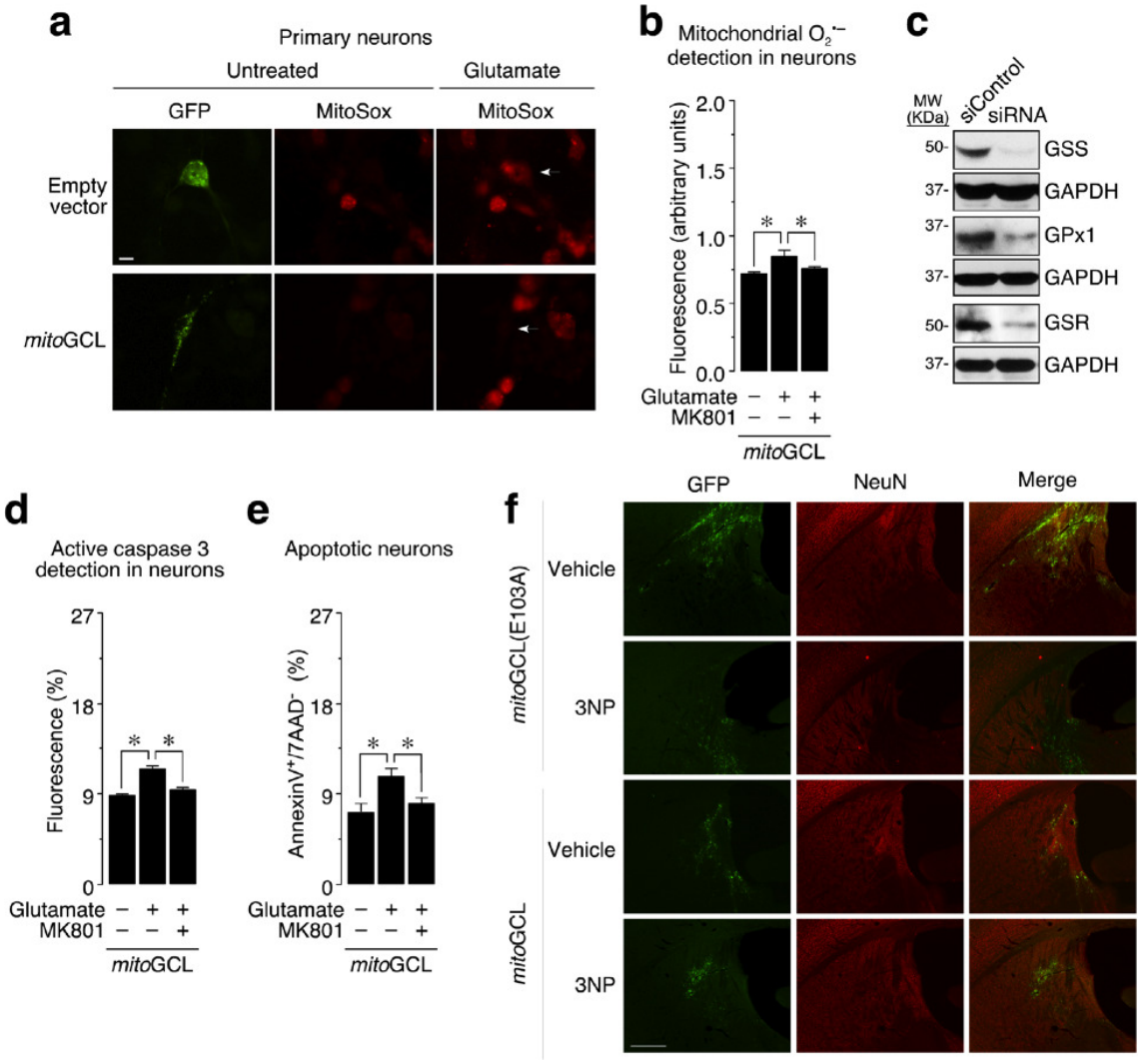
Supplementary Information

Supplementary Figures



Supplementary Figure S1. Glutathione redox status and ROS detection by mitoGCL expression in HEK23T cells. (**a**) Expression of *mito*GCL did not alter cytosolic GSH concentrations in HEK203T cells. (**b**) The increase in mitochondrial H_2O_2 detection caused by rotenone (10 µM/15 min) was prevented by *mito*GCL, as judged by the fluorescence emitted from the expression of the mitochondrial-tagged plasmid probe, pHyPer-dMito, in intact HEK203T cells; scale bar = 50 µm. (**c**) Measurement of the rate of H_2O_2 detection by the AmplexRed method evidenced significant decrease in detected H_2O_2 in intact HEK293T cells. (**d**) Rotenone (Rot., 1 µM/4 h) decreased the reduced form of glutathione (GSH), and increased its oxidized form (GSSG) that was prevented by expression of *mito*GCL in HEK293T cells (Untr.=untreated). (**e**) Total cellular GSH concentration was efficiently decreased 3 days after transfection of HEK293T cells with an shRNA against GCL (shGCL) in the presence of either wild type *mito*GCL or the shGCL-refractory form, *mito*GCL(mut). (**f**) Rotenone (10 µM) increased mitochondrial ROS, as judged by MitoSox fluorescence in HEK293T cells; this effect was abolished by GSS silencing and enhanced by GPx1 knockdown. **P*<0.05; $^{\#}$*P*<0.05 *versus* siControl (ANOVA). All data are expressed as mean ± S.E.M (n = 3–4).



Supplementary Figure S2. ROS detection, siRNA efficacy, and cell death by *mito*GCL-treated as assessed in primary neurons and in mice *striatum in vivo.* (**a**) Real-time fluorescence imaging of rat primary neurons incubated with the mitochondrial probe, MitoSox, revealed increased ROS detection by glutamate treatment (40 µM/15 min) in efficiently transfected neurons (GFP^+) with the empty vector, but not with the vector expressing *mito*GCL; scale bar = 10 µm. (**b**) Blockade of N-methyl-D-aspartate (NMDA) receptor with its antagonist, MK801 (1 µM) prevented the increase in mitochondrial ROS (MitoSox fluorescence). (**c**) Western blots showing the efficacy of siRNA duplexes, used at 100 nM for 3 days in primary neurons, against glutathione synthetase (GSS), glutathione peroxidase-1 (GPx1) and glutathione reductase (GSR); control siRNA (siControl) was an siRNA against luciferase. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as loading control. (**d**) Blockade of N-methyl-D-aspartate (NMDA) receptor with its antagonist, MK801 (1 µM) prevented the increase in active caspase-3 neurons, and (**e**) Blockade of N-methyl-D-aspartate (NMDA) receptor with its antagonist, MK801 (1 µM) prevented the increase in apoptotic (annexin V^+/7-AAD^-) neurons, triggered by the incubation with glutamate

(100 μM/5 min). (**f**) Fluorescence images showing neuronal loss by 3NP treatment and prevention by *mito*GCL. Lentiviral particles expressing wild type or inactive (E103A) *mito*GCL were stereotaxically injected ($5x10^6$ p.f.u./3 μl at 0.25 μl/min) into the *striatum* of adult mice. After three days, 3NP was intraperitoneally injected (seven doses of 50 mg/kg, twice per day), which induced, 3 days later, a significant loss of striatal $NeuN^+$ neurons only in the mice that received inactive *mito*GCL(E103A), but not in those that received wild type *mito*GCL; mice treated with vehicle instead of 3NP showed no neuronal loss regardless of the isoform of *mito*GCL (wild type or inactive) lentiviral particles injected; the quantitative analysis is shown in Figure 4d; scale bar = 500 μm. *P<0.05 (ANOVA). All data are expressed as mean ± S.E.M (n = 3–4).

Supplementary Tables

Supplementary Table S1. Concentrations of reduced and oxidized γ-glutamylcysteine, and reduced glutathione, after GSS knockdown in HEK293 cells.

	Reduced γ-glutamylcysteine (nmol/mg protein)	Oxidized γ-glutamylcysteine	GSH (nmol/mg protein)
siControl	0.32±0.01	ND	23.5±0.8
siGSS	0.37±0.02*	ND	13.3±1.3*
siGSS+*mito*GCL	0.41±0.02*	ND	13.9±0.2*

Results are mean ± SEM values (n=8-10); *p<0.05 *versus* siControl (ANOVA); ND, not detected.

Supplementary Table S2. Concentrations of reduced and oxidized forms of γ-glutamylcysteine, and reduced glutathione in different tissues in adult male mice *in vivo*.

	Reduced γ-glutamylcysteine (nmol/mg protein)	Oxidized γ-glutamylcysteine (pmol/mg protein)	GSH (nmol/mg protein)
Brain	0.26±0.01	1.46±0.25	24.2±1.4
Liver	0.41±0.02	10.63±1.87	63.9±5.2
Kidney	1.66±0.36	1.14±0.25	20.7±3.2

Results are mean ± SEM values (n=7-8 mice).

Supplementary Table S3. Thiol-redox modification of proteins by γ-glutamylcysteine or glutathione after expression of *mito*GCL in HEK293 cells.

	By γ-glutamylcysteine	By glutathione (pmol/mg protein)
Empty vector	ND	5.69±0.56
*mito*GCL	ND	5.03±0.36

Results are mean ± SEM values (n=3); ND, not detected.

Supplementary Methods

Cell transfections and treatments. Transfections of primary neurons with siRNAs were performed at day 3 *in vitro*, although experiments and sample collection were done at day 6. In all siRNA-mediated knockdown experiments, a preliminary set of transfections were performed using a range of 20 to 100 nM of each siRNA to obtain a concentration-dependent knockdown effect; however, the results shown correspond to those experiments using 100 nM of each siRNA. Transfections of primary neurons with plasmid vectors were performed at day 4 or 5 *in vitro*. In these, we used 0.4–1.6 μg ml^{-1} of each plasmid. All transfections in neurons were performed using Lipofectamine2000 (Invitrogen, Madrid, Spain), following the manufacturer's instructions, also described elsewhere[37]. After 6 h, the medium was removed and cells were further incubated, for the times indicated in the figures, in the presence of culture medium. For plasmids, the transfection efficiencies in neurons were, approximately, 7%, as judged by the proportion of GFP^+ neurons versus total number of neurons (as evidenced by DAPI-staining); for siRNA duplexes, the transfection efficiencies were >90%, as judged by the level of protein knockdown shown in the corresponding figures. For experiments involving glutamate-receptor stimulation, neurons were incubated at day 6 *in vitro* (i.e., 1-3 days after transfections, when applicable) with 100 μM glutamate (in the presence of 10 μM glycine) in buffered Hank's solution (134.2 mM NaCl, 5.26 mM KCl, 0.43 mM KH_2PO_4, 4.09 mM $NaHCO_3$, 0.33 mM Na_2HPO_4, 5.44 mM glucose, 20 mM HEPES, 4 mM $CaCl_2$, pH 7.4) for 5 min, following a standard procedure[38], washed, and further incubated for 24 h post-stimulation (unless otherwise specified) in DMEM. To block N-methyl-D-aspartate (NMDA) receptor, neurons were pre-incubated with 10 μM of MK801 (Sigma) 5 min before glutamate additions. In some experiments, instead of glutamate, neurons were incubated in the presence of rotenone (Rot, 10 μM), antimycin A (AA, 10 μM) or 3 nitropropionic acid (3-NP, 2 mM). For HEK293T cell treatment, cells were seeded at $1x10^5$ cells/cm^2 or $5x10^4$ cells/cm^2 for experiments involving nucleic acid transfections. In these cases, the cells were used 24 h after transfections with plasmids or 3 days after transfections with siRNAs. For siRNAs, we used Lipofectamine2000 (Invitrogen) following the manufacturer's instructions, and for plasmids, we used 1 μM polyethylenimine (PEI, Sigma)[39] with some modifications. Briefly, a mix of PEI with the corresponding DNA plasmid was added to cells after an

incubation step of 10 min at room temperature in Optimem medium (Invitrogen). Cells were then incubated with this PEI/DNA-containing solution at 37ºC during 1 h, followed by washing with PBS and incubation in DMEM supplemented with either 10% or 0.05% FCS (v/v). In some experiments, HEK293T cells were incubated with rotenone (1 µM for 4 h, or 10 µM for 15 or 45 min, as indicated) 3-NP (10 mM for 2 h).

Subcellular fractionation. To fractionate cytosol from intact mitochondria, we used a differential centrifugation protocol[40]. In brief, cells grown in 145 cm^2 dishes (Nunc, Thermo Fisher Scientific, Roskilde, Denmark) were collected in mitochondria isolation buffer (MIB, 320 mM sucrose, 1 mM potassium EDTA, 10 mM Tris-HCl and the protease inhibitor cocktail (2 mM AEBSF, 0.3 µM Aprotinin, 130 µM Bestatin, 1 mM EDTA, 14 µM E-64, 1 µM Leupeptin, catalog number P2714, Sigma) at pH 7.4. Cells were centrifuged at 600x*g* for 5 min at 4°C and the pellet re-suspended in MIB. After a 18 stroke homogenization step in a tight-fitting glass-teflon homogenizer, homogenate was centrifuged three times at 1300x*g* for 10 min at 4°C, keeping the supernatants in every step. The mitochondrial pellet that resulted from the final centrifugation (at 17,000x*g* for 12 min at 4°C) was re-suspended in MIB (for reactive oxygen species and enzymatic analysis) or RIPA buffer (see composition below under western blotting subsection), and the supernatant was kept on ice (cytosolic fraction). To obtain reproducible results, only enriched mitochondrial and cytosolic fractions were used for the experiments, which were always assessed by the analysis of citrate synthase or lactate dehydrogenase activities, as previously described[40].

Western blotting. Cells or subcellular fractions were lysed in RIPA buffer (2% sodium dodecylsulphate, 2 mM EDTA, 2 mM EGTA and 50 mM Tris pH 7.5), supplemented with phosphatase inhibitors (100 µM phenylmethylsulfonyl fluoride, 50 µg/ml antipapain, 50 µg/ml pepstatin, 50 µg/ml amastatin, 50 µg/ml leupeptin, 50 µg/ml bestatin, 1 mM o-vanadate, 50 mM NaF, and 50 µg/ml soybean trypsin inhibitor) and boiled for 5 min. Extracts were centrifuged at 13,000x*g* for 5 min at 4°C, and aliquots of lysates (20-90 µg protein) were subjected to sodium docedyl sulfate-polyacrylamide (SDS-PAGE) electrophoresis on a 8, 10 or 12% acrylamide gel (MiniProtean®, Bio-Rad) including BenchMark™ (Invitrogen) or Dual Colors™ (BioRad) as prestained protein ladders. The resolved proteins were transferred electrophoretically to nitrocellulose membranes (Hybond-ECL, Amersham Bioscience Europe GmbH,

Barcelona, Spain). Membranes were blocked with 5% (w/v) low-fat milk in 20 mM Tris, 500 mM NaCl, and 0.1% (w/v) Tween 20, pH 7.5, for 1 h. After blocking, membranes were immunoblotted with primary antibodies at dilutions ranging from 1:500 to 1:1000, unless otherwise stated, overnight at 4ºC. After incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000 dilution, Pierce, Thermo Fischer Scientific) or goat anti-mouse IgG (1:1000 dilution, BioRad), membranes were immediately incubated with the enhanced chemiluminescence kit SuperSignal West Dura (Pierce, Thermo Scientific, Illinois, USA) for 5 min, before exposure to Kodak XAR-5 film for 1-5 min, and the autoradiograms scanned.

***In vitro* H_2O_2 quantification.** To assess the *in vitro* reaction of GSH or γGC with H_2O_2, we used a previously described protocol[41]. GSH or γGC (at concentrations ranging from 0 to 2000 μM) were incubated with H_2O_2 (100 μM), either in the absence or in the presence of 5 U/ml of GPx1 purified from bovine erithrocytes (catalog number 3167, Sigma), and/or (as indicated) 5 U/ml of GSR from bakers yeast (catalog number 3664, Sigma) in reaction buffer (0.1 M $NaHPO_4$, 1 mM EDTA diK^+, 0.4 mM NADPH, pH 7.4) at 25ºC. The reaction was stopped every 1 min by the addition of 11.4 mM HCl. Then, 3.2 mM amonium ferrous sulfate hexahydrate $(NH_4)_2Fe(SO_4)_2$ and 180 mM KSCN (final concentrations) were added, and the absorbance at 492 nm after 10 min of incubation at room temperature was measured, in a 96-well plate reader (Multiscan Ascent, Thermolabsystems, Belgium). For the calculations, we used a standard curve using 0-240 μM H_2O_2.

Determination of lactate dehydrogenase and citrate synthase activities. These were determined as described[40] using 10 μg of protein in a final volume of 1 ml. Lactate dehydrogenase was determined following the rate in the change of absorbance at 340 nm in a 80 mM Tris buffer containing 200 mM NaCl and 0.28 mM NADH at pH 7.5 and at 30ºC. Citrate synthase was determined following the rate in the change of absorbance at 412 nm in a 100 mM Tris-HCl buffer containing 0.2 mM oxaloacetate, 0.1 mM acetyl-CoA, 0.2 mM DTNB and 0.1% (v/v) Triton X-100, at pH 8.0 and at 30ºC.

Determination of glutathione concentrations. This was performed by both the enzymatic and the HPLC (with electrochemical detection) methods. For the enzymatic method, 1% (wt/vol) of sulfosalicylic acid was added to cells or isolated mitochondria, the cell lysates were centrifuged at 13,000x*g* for 5 min at 4°C, and the supernatants were used for the determination of total glutathione (GSH + 2xGSSG), using GSSG (0-50 μM) as standard, as described previously[42]. Total glutathione was measured in reaction buffer (0.1 mM $NaHPO_4$, 1 mM EDTA, 0.3 mM DTNB, 0.4 mM NADPH, glutathione reductase 1 U/ml, pH 7.5) by recording the increase in the absorbance after the reaction of GSH with DTNB. GSSG was measured (either under basal conditions or after incubation of cells with 10 μM rotenone for 3h at 37ºC in Hank's buffer). GSSG was quantified after derivation with 2-vinylpiridine (Sigma) plus 0.2 M Tris (pH 5-7). Absorbance at 405 nm was recorded for 2.5 min in 15 s intervals using a (Multiscan Ascent, Thermolabsystems, Belgium) plate reader. GSH concentration values were confirmed by the HPLC method; in this, ice-cold ortho-phosphoric acid (15 mM, final concentration) was added to cells, cytosol or isolated mitochondria, and then subjected to HPLC with electrochemical detection (upstream electrode: +100 mV; downstream electrode: +100 to +650 mV (in +50 mV increments)[43] and an external GSH standard (Sigma).

Samples preparation for the determination of thiols and disulfides. For the assessment of free reduced and oxidized forms low molecular weight thiols, we used a previously reported protocol[44] with some modifications. Cells were seeded in 60 cm^2 at a density of 5 x 10^4 cells/cm^2. After 4 days in culture, cells were rinsed with PBS and incubated with Hank's buffer (5.5 mM glucose, pH 7.4) for 7 h at 37ºC in a 5% CO_2 atmosphere. Then, cells were washed with ice-cold PBS containing 10 mM N-ethylmaleimide (NEM, Sigma) for 2 min and then scraped in 400 μl of the PBS-NEM mixture. For in vivo analyses, mice were anesthesized with isofluorane (Abbot) and sacrificed by cervical dislocation; brain, liver and kidney were rapidly deep-frozen (freeze-clamping) in liquid nitrogen. Aliquots of 100 mg of tissue were homogenized in 400 μl of 10 mM NEM. In all cases, after addition of 4% (v/v) perchloric acid, samples were centrifuged at 13,000x*g* for 10 min at 4ºC, and the supernatant collected and stored at -80ºC until thiols determination by ultra performance liquid chromatography and mass spectrometric detection (UPLC-MS/MS).

Samples preparation for the determination of protein disulfides formed with low molecular weight thiols. Cells were seeded as indicated in the previous paragraph for free thiols and disulfides measurement. After washing with ice-cold PBS containing 10 mM NEM for 2 min, cells were detached with 10% trichloroacetic acid (TCA) and centrifuged at 10,000x*g* for 2 min at 4ºC. The pellet was washed twice with 10% TCA and dissolved in 50 mM HEPES with 2% SDS, pH 8, with powder $NaHCO_3$ added until saturation. Once re-suspended, an aliquot of the pellets was used for the determination of protein concentration. Another aliquot was taken and treated with 2.5 mM dithiothreitol (DTT, Sigma). After 1 h incubation at 40ºC in agitation and verified the pH to be between 9-10, 10 mM NEM was added and vortex manually. Perchloric acid (4%) was then added, and samples centrifuged at 10,000x*g* for 10 min at 4ºC. Samples were stored at -80ºC until analyzed by UPLC-MS/MS.

Ultra performance liquid chromatography and mass spectrometry (UPLC-MS/MS). This was carried out using a triple-quadruple mass spectrometer (Waters, Manchester, UK) equipped with Xevo TQ-S. Analytical separation was carried out at 50ºC using a core shell C19 Kinetex column (100 x 2mm, 3 µm, Phenomenex) using an injection volume of 2 µl. A 6 min gradient elution was performed at a flow rate of 400 µm min^{-1} as follows: initial conditions A (water, 0.1% v/v HCOOH) 100%, B (acetonitrile, 0.1% v/v HCOOH) were kept for 1 min, followed by a linear gradient up to 85% B in 1.5 min, then isocratic condition was held for 2 min. Finally, a 0.25 min linear gradient was used to return to the initial conditions, which were held for 1.25 min to re-equilibrate the system. Positive ion electrospray tandem mass spectra were recorded using the following conditions: capillary voltage 3 kV, source temperature 350ºC, cone and nebulisation gases were set at 750 and 180 L/h, respectively.

Flow cytometric analysis of apoptotic cell death. Cells were carefully detached from the plates using 1 mM EDTA (tetrasodium salt) in PBS (pH 7.4). APC-conjugated annexin-V and 7-amino-actinomycin D (7-AAD) (Becton Dickinson Biosciences) were used to determine quantitatively the percentage of apoptotic neurons by flow cytometry. Cells were stained with annexin V-APC and 7-AAD in binding buffer (100 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$), according to the manufacturer's instructions, and 3 x 10^5 cells were analysed, in four replicates per condition, on a FACScalibur flow cytometer (15 mW argon ion laser tuned at 488 nm; CellQuest software, Becton

Dickinson Biosciences). GFP^+ and GFP^- cells were analysed separately, and the annexin V-APC-stained cells that were 7-AAD-negative were considered to be apoptotic[45]. The analyzer threshold was adjusted on the flow cytometer channel to exclude most of the subcellular debris in order to reduce the background noise owing to the neurite disruption during neuronal detaching. Data were expressed as percentages.

Flow cytometric detection of active caspase-3. Active caspase-3 was detected using the ApoActive3™ Kit (Bachem, San Carlos, CA, USA), following the manufacturer's instructions. After detaching cells with 1 mM EDTA (tetrasodium salt) and centrifuged, cell pellets were fixed during 20 min, re-suspended in PBS + 2% bovine serum albumin (BSA) and incubated for 1 h with 1X rabbit anti-caspase 3. Cells were then incubated with 1:500 anti-rabbit CyC3 (Jackson Immunoresearch, Pennsylvania, U.S.A.) for 1 h. Between each step, cells were washed with either PBS (until labelling of samples) or PBS + 1% BSA, and re-suspended in PBS + 2% BSA before analysis by flow cytometry (tuned at 488 nm; CellQuest software, Becton Dickinson Biosciences).

Stereotaxic injection of lentiviral particles. Stereotaxic injections were performed as previously described[46], with some modifications. Male mice (10-16 weeks old) were anesthetized by inhalatory induction and maintained with sevofluorane (Sevorane. Abbot), using a gas distribution column (Hersill H-3, Madrid, Spain) and a vaporizer (InterMed Penlons Sigma Delta, OX, UK). Mice were placed in a stereotaxic alignment system (Model 1900, David Kopf Instruments, CA, USA) with digital read out (Wizard 550, Anilam, NY, USA) and complemented with a stereomicroscope (Nikon SMZ 645, Tokyo, Japan) and a fibre optic cold light source (Schott KL1500 compact, Mainz, Germany). Injection was performed into both hemispheres at coordinates: 0.5 mm anterior to bregma, 2.1 mm lateral to midline, and 3.8 mm ventral to dura[47] using a 5-µl Hamilton syringe (Microliter 65RN, Hamilton, NV, USA) with a 26 S needle (type 2 tip). Lentiviral particles were delivered at a concentration of 5×10^6 total plaque forming units in 3 µl of PBS with a mini-pump (UltraMicroPump III, World Precision Instruments, USA) and a digital controller (Micro4 (UMC4) World Precision Instruments, USA), during 12 min at a rate of 0.25 µl/min. The syringe was left in place for 10 min before slowly retracting it to allow for lentivirus infusion and to prevent reflux. Wounds were irrigated with 0.9% sterile saline solution and animals were

allowed to recover from anaesthesia in cages placed on a 37ºC thermostatted plate (Plactronic Digital, 25x60, JP Selecta, Barcelona, Spain).

Induction of *in vivo* neurodegeneration by 3-nitropropionic acid (3NP). Three days after stereotaxic lentiviral partciles injection, *in vivo* neurodegeneration was induced in mice (strain C57BL6/J) following a previously reported protocol[48], with some modifications. In brief, 3NP (25 mg/ml) was dissolved in PBS, adjusted to pH 7.4 with 1 M NaOH and maintained at 4°C for up to 1 week. Mice received *i.p.* injections (50 mg/kg of body weight) of 3NP or vehicle (PBS) every 12 h for a total of seven injections in a volume of 200 µl. Six to eight hours after the last dose, mice were perfused for inmunhistochemistry.

Tissue sampling for biochemical analyses. Mice (C57BL6/J) were anaesthetized with xylacine hydrochloride and ketamine hydrochloride (4:1) and sacrificed 6 days after stereotaxic administration of lentiviral particles. After decapitation, brains were extracted and dissected out coronally in an adult mouse matrix (ASI instruments, MI, USA), collecting the tissue comprised between lines 7 and 11 (1 mm/line, caudal-rostral sense) of the matrix and verifying the presence of the scares derived from stereotaxic surgery in the recollected fraction. After removal of the cortex in each hemisphere, the remaining tissue was excised in two parts, for determination of GSH concentration and GCL activity from the same tissue, and each fragment was weighed. Tissue was washed twice with PBS, and 10 µl/mg tissue of 1% (wt/vol) of sulfosalicylic acid, or isolation medium (320 mM sucrose, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) was added for the determination of GSH or GCL activity, respectively. Tissue was homogenized in a tight-fitting glass-teflon homogenizer (20 strokes) and centrifuged at 13,000x*g* for 15 min at 4ºC, keeping the supernatants for the determinations.

Immunocytochemistry. Cells grown on glass coverslips or µ-slide 8 well plastic bottom dishes (Ibidi, Martinsried, Germany) were fixed with 4% paraformaldehyde (vol/vol, in PBS) for 30 min, rinsed with PBS and permeabilized for 5 min with 0.3% Triton X-100. Cells were then incubated for 30 min at room temperature in PBS containing blocking solution (0.1% triton X-100, 5% horse serum) and kept overnight at 4°C with anti-GCL antibody (1:40 dilution)[49]. After washing with PBS, cells were incubated with the secondary antibody Alexa 488-conjugated goat anti-rabbit (1:400

dilution; Molecular Probes, Invitrogen) for 1 h at room temperature. Cells transfected with pMitoDsRed2 plasmid vector (Clontech) expressed red fluorescent protein in mitochondria, whereas cells transfected with pIRES2-EGFP plasmid vector revealed diffused GFP fluorescence in the cytosol. Dishes and coverslips were washed, and coverslips were mounted in SlowFade® light antifade reagent (Molecular Probes, Oregon, USA) on glass slides for phase-contrast or fluorescence microphotographs at x40 or x60 magnification.

Inmunohistochemistry and determination of lentiviral transduction efficiency and neuronal loss. Mice (C57BL6/J) were deeply anaesthetized by i.p. injection of a mixture (1:4) of and xilacine hydrochloride (Rompún; Bayer, Kiel, Germany) and ketamine hydrochloride/chlorbutol (Imalgene; Merial, Lyon, France), using 1 ml of the mixture per kg of body weight, and then perfused intra-aortically with 0.9% NaCl, followed by 5 ml/g per body weight of Somogy's fixative (4% (p/v) paraformaldehide, 0.2% (p/v) picric acid in 0.1 M phosphate buffer (PB) (pH 7.4). After perfusion, brains were dissected out coronally in two parts and post-fixed, using the Somogy's fixative, for overnight at 4 ºC. Brain blocks were then rinsed successively for 10 min, 30 min, and 2 h with 0.1 M PB solution (pH 7.4), and immersed in 20% and 30% (w/v) sucrose in PB solution sequentially until they sank. After cryoprotection, 40 µm-thick coronal sections were obtained with a freezing-sliding cryostate (Leica CM 1950 AgProtect, Leica, Nussloch, Germany). The sections were collected in 0.05% sodium azide (wt/vol) in 0.1 M PB. Coronal sections were rinsed in 0.1 M PB three times each for 10 minutes, and then incubated sequentially in: (i) 5 mg/ml sodium borohydride in PB for 30 minutes to remove aldehyde autofluorescence; (ii) 1:1000 rabbit anti-GFP (ab290, Abcam) and 1:1000 mouse anti-NeuN (MAB377, Chemicon international. Temecula, CA, USA), in 0.2% Triton X-100 (Sigma) and 5% normal goat serum (Jackson Immoresearch, Pennsylvania, U.S.A.) for 72 h at 4 ºC in 0.1 M PB; (iii) 1:500 Alexa 488-conjugated goat anti-rabbit and 1:500 Alexa 594-conjugated goat anti-mouse (Molecular Probes, Invitrogen, Oregon, USA) for 2 h at room temperature; (iv) 0.5 µg/ml DAPI in PB for 10 minutes at room temperature. Except between the staining with secondary antibodies and DAPI, after each step the sections were carefully rinsed three times each for 10 min in PB. After rinsing, sections were mounted with Fluoromount (Sigma) aqueous mounting medium. Sections were examined with epifluorescence and appropriated filters sets using a microscope (Nikon Inverted

microscope Eclipse Ti-E, Japan) equipped with a pre-centered fiber illuminator (Nikon Intensilight C-HGFI, Tokyo, Japan) and B/W CCD digital camera (Hamamatsu ORCA-ER, Hamamatsu, Japan), or a confocal microscope (TCS SP2; Leica, Mannheim, Germany). To determine the efficiency of transduction of the lentiviral particles, vehicle +*mito*GCL-treated mice (n=4) were used. An area of 0.5 mm^2 in the injection zone was counted in 3 striatal slices/mouse. A 8.8±1.6% of the striatal neurons, identified by NeuN staining (610±60 $NeuN^+$ cells per slice) were GFP^+ (efficiently transduced). In addition, a 40.8±5.7% (mean±SEM) of cells that were efficiently transduced (i.e., GFP^+) in the striatum were found to be $NeuN^+$. To determine neuronal loss, the number of $NeuN^+$ cells was counted in a peri-injection area of 0.5 mm^2 in 3 striatal slices/mouse.

TUNEL assay and neuronal loss. Apoptotic cell death after *in vivo* treatments was assessed by the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assay (Roche Diagnostics). Brain sections, fixed as above, were pre-incubated in TUNEL buffer containing 1 mM $CoCl_2$, 140 mM sodium cacodylate and 0.3% Triton X-100 in 30 mM Tris buffer, pH 7.2, for 30 min. After incubation at 37°C with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase (800 U/ml) and nucleotide mixture (1 μM) for 90 min, sections were rinsed with PBS and counterstained with Cy3-streptavidin (Jackson Immunoresearch Laboratories). Each data point was obtained by counting $TUNEL^+/NeuN^+$ cells in a 0.3 mm^2 area in three striatal slices.

Rotarod test. Motor balance and coordination was analysed using the rotarod test as described[50] once daily during days 1, 2 and 3 of 3NP treatment. Mice were trained for 3 days before the stereotaxic surgery. All determinations were carried out at the same time every day. Mice were allowed to stay for 300 s on a five-lane accelerating rotarod (Model 47600. Ugo Basile, Comerio, Italy) rotating rod with a continuous accelerating rotation speed from 4 to 40 rpm, increasing 4 rpm every 30 s and reaching the final speed at 270 s. Latency to fall was measured during this period, annotating the time the animal stayed on the rotation rod. Data from tests in which animals completed 3 turns without walking were disregarded.

Supplementary References

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