

Knockdown of Glutamate-Cysteine Ligase by Small Hairpin RNA Reveals That Both Catalytic and Modulatory Subunits Are Essential for the Survival of Primary Neurons*

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Juan I. Diaz-Hernandez^{†§1}, Angeles Almeida^{†§1,2}, Maria Delgado-Esteban[§], Emilio Fernandez[§], and Juan P. Bolaños^{§3}

From the [†]Unidad de Investigación, Hospital Universitario de Salamanca and the [§]Departamento de Bioquímica y Biología Molecular and the Centro Nacional de Investigaciones Cardiovasculares, Universidad de Salamanca, Salamanca 37007, Spain

Glutathione deficiency is an early biochemical feature that occurs during apoptotic neuronal death associated with certain neurological disorders such as Parkinson disease. However, whether specific targeting of glutathione biosynthesis in neurons is sufficient to trigger neurodegeneration remains undetermined. To address this issue, we used a vector-based small hairpin RNA (shRNA) strategy to knock down each subunit of glutamate-cysteine ligase (GCL; γ -glutamylcysteine synthetase), the heterodimeric enzyme that catalyzes the rate-limiting step of glutathione biosynthesis. Independent targeting of the catalytic and modulatory subunits by shRNA caused disruption of GCL as assessed by Northern and Western blotting, enzyme activity, and glutathione concentrations. Silencing each subunit in primary cortical neurons spontaneously elicited time-dependent apoptotic death, an effect that was synergistic with glutamate or nitric oxide treatment. Moreover, neuronal apoptosis by GCL knockdown was rescued by expressing the corresponding subunit full-length cDNA carrying silent mutations within the shRNA target cDNA sequence and by incubating neurons with γ -glutamylcysteine or glutathione ethyl ester. In contrast, supplying glutathione precursors to neurons from co-cultured astrocytes did not prevent the apoptotic death triggered by GCL knockdown. Finally, overexpressing the catalytic (but not modulatory) GCL subunit full-length cDNA increased enzyme activity and glutathione concentrations, yielding neurons more resistant to glutamate- or nitric oxide-mediated apoptosis. Thus, specific and independent disruption of each subunit of GCL in neurons can be said to cause a primary decrease in glutathione that is sufficient to promote neurodegeneration.

Glutathione is a critical non-protein thiol involved in antioxidant defense (1) and whose loss has been associated with neurodegeneration (2). Thus, glutathione deficiency is the earliest known biochemical indicator of nigrostriatal degeneration in Parkinson disease (3), an observation suggesting that oxidative stress may be a cause of the apoptotic neuronal death associated with neurodegenerative processes and aging (2, 4–6). Unfortunately, to date, no unambiguous demonstration that

the specific loss of glutathione is sufficient to trigger neurodegeneration has been reported.

Glutathione is a tripeptide (γ -glutamylcysteinylglycine) synthesized by two consecutive ATP-dependent reactions. Glutamate-cysteine ligase (GCL⁴; EC 6.3.2.2, γ -glutamylcysteine synthetase) catalyzes the first and rate-limiting step, forming γ -glutamylcysteine from glutamate and cysteine (7). This is followed by the glutathione synthetase (EC 6.3.2.3)-catalyzed reaction, which binds glycine to γ -glutamylcysteine, forming glutathione (8). GCL is a heterodimeric enzyme composed of a catalytic subunit (heavy; 73 kDa) (9) and a modulatory subunit (light; 27.7 kDa) (10). Studies performed with purified GCL suggest that the active site resides at the catalytic subunit, whereas the modulatory subunit increases the affinity of the catalytic subunit for glutamate and decreases the sensitivity to feedback inhibition by glutathione (9). Homozygous embryonic mice with targeted disruption of the catalytic GCL subunit (Cat) have been obtained, but they are not viable beyond the 8th gestational day (11, 12). On the other hand, initial characterizations of homozygous knockout mice for the modulatory GCL subunit (Mod) revealed that they are viable and fertile, hence providing an interesting model for the *in vivo* study of mild and chronic glutathione deficiency (13). However, the biochemical, molecular, and cellular phenotypes of the brains of these mice have not yet been investigated. Thus, the specific contribution of each GCL subunit to the survival of neurons is still elusive.

To disrupt cellular glutathione biosynthesis, a widely used pharmacological approach has long been the administration of L-buthionine sulfoximine, a competitive inhibitor of GCL activity that presumably interferes exclusively with the catalytic subunit (14). However, L-buthionine sulfoximine also inhibits the glutamyl amino acid transporter (15) and hence may affect other unknown biochemical pathways. An elegantly designed inducible vector-based full-length antisense cDNA approach has been described to induce a concomitant depletion of both GCL subunits in PC12 cells (16, 17). Such an approach requires cell lines constitutively expressing the tetracycline repressor, but is unsuitable for primary cells such as cortical neurons. In addition, this system does not afford any information about the impact of each GCL subunit on glutathione biosynthesis.

With the aim of elucidating the specific contribution of each GCL subunit to glutathione biosynthesis and neuronal survival by overcoming these potential drawbacks, in this work, we used the small interfer-

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¹ Both authors contributed equally to this work.

² Supported by Fondo de Investigación Sanitaria Grant FIS03/1055 and Junta de Castilla-León Grant SA082A05. To whom correspondence should be addressed: Unidad de Investigación, Hospital Universitario de Salamanca, Paseo de San Vicente 58-182, 37007 Salamanca, Spain. Tel.: 34-923-294-526; Fax: 34-923-294-579; E-mail: aaparra@usal.es.

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⁴ The abbreviations used are: GCL, glutamate-cysteine ligase; Cat, catalytic (heavy) glutamate-cysteine ligase subunit; Mod, modulatory (light) glutamate-cysteine ligase subunit; siRNA, small interfering RNA; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; shRNA, small hairpin RNA; RNAi, RNA interference; GFP, green fluorescent protein; HEK293T, human embryonic kidney 293T; DETA/NO, (Z)-1-[2-aminoethyl-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate; 7-AAD, 7-amino-actinomycin D.

ing RNA (siRNA) strategy. This system, which has been proven to be more specific and efficient than the full-length antisense cDNA strategy (18), allowed us to identify siRNAs (delivered from a plasmid expression vector) that triggered highly specific and efficient GCL subunit protein disruption and the inhibition of enzyme activity, leading to glutathione depletion and neuronal death. We believe that these results unambiguously confirm that *both* GCL subunits are essential for glutathione biosynthesis and neuronal survival.

MATERIALS AND METHODS

Reagents—Dulbecco's modified Eagle's medium (DMEM), poly-D-lysine, horse serum, cytosine arabinoside, and glutathione ethyl ester were obtained from Sigma. DMEM was always supplemented with penicillin (100 units/ml), streptomycin (100 μ g/ml), and amphotericin B (0.25 μ g/ml) (Sigma). Fetal calf serum (FCS) was purchased from Roche Diagnostics (Heidelberg, Germany). The apoptosis assay kit was purchased from BD Biosciences. Lipofectamine 2000 was purchased from Invitrogen (Groningen, The Netherlands). γ -Glutamylcysteine was purchased from Bachem (Weil am Rhein, Germany). Other substrates, enzymes, and coenzymes were purchased from Sigma, Roche Diagnostics, Merck (Darmstadt, Germany), and Promega Biotech (Madrid, Spain). Plastic tissue culture flasks were purchased from Nunc (Roskilde, Denmark).

Design of Small Hairpin RNAs (shRNAs) for GCL—GCL subunit knockdown was achieved by RNA interference (RNAi) using a vector-based shRNA approach (19). Five shRNA target cDNA sequences were selected for each GCL subunit according to a previously reported rational design protocol (see TABLE ONE) (20). As a control, we used the firefly luciferase-targeted oligonucleotide 5'-CTGACGCGGAATAC-TTTCGA-3' as reported previously (21). All sequences were BLAST-confirmed for specificity. Synthetic forward and reverse 64-nucleotide oligonucleotides (Isogen Life Science, Maarsen, The Netherlands) were designed, annealed, and inserted into the BglII/HindIII sites of the pSUPER-neo/GFP vector (OligoEngine, Seattle, WA) following the manufacturer's instructions. These constructions express 19-bp 9-nucleotide stem-loop shRNAs targeted against GCL, the catalytic (Cat shRNA) or modulatory (Mod shRNA) GCL subunit, or luciferase (control shRNA) mRNAs. The concomitant expression of green fluorescent protein (GFP) from this vector allowed the identification of transfected cells by fluorescence microscopy and flow cytometry.

Plasmid Constructions—The Cat full-length cDNA (2023 bp; GenBankTM accession number NM_012815) was obtained by ligating, at position 1117 of the cDNA (with a unique ClaI site), two cDNA fragments of \sim 1 kb each, which were independently obtained by reverse transcription-PCR of total RNA from rat astrocytes as template. The oligonucleotides used were 5'-CCGGAATTCGCCATGGGGCT-GCTG-3' (sense) and 5'-TGCCAGAAGGTGATCGATGCCTT-3' (antisense) for the 5'-end fragment and 5'-AAGGCATCGATCACCT-TCTGGCA-3' (sense) and 5'-CCGGAATTCCTCAACTGGAACACTG-TAG-3' (antisense) for the 3'-end fragment (with EcoRI sites underlined). The Mod full-length cDNA (905 bp; GenBankTM accession number NM_017305) was obtained by reverse transcription-PCR of total RNA from rat astrocytes as template. The oligonucleotides used were 5'-CGCGGATCCTCCCTCGGGCGGCAGCT-3' (sense) and 5'-CGCGGATCCTAAATACAAGGCCCTGAG-3' (antisense) (with BamHI sites underlined). The reverse transcription-PCR conditions were as follows: 50 min at 48 °C for reverse transcription; 3 min at 94 °C; 40 cycles of 1 min at 94 °C, 1 min at 53 °C for Cat and at 55 °C for Mod, and 2.5 min for Cat and 1 min for Mod at 68 °C; and a final extension for 10 min at 68 °C. The cDNA products were purified,

digested, and subcloned into the corresponding EcoRI or BamHI site of the pIRES2-EGFP mammalian expression vector (Invitrogen) (pIRES-Cat or pIRES-Mod) and sequenced. The concomitant expression of GFP from this vector allowed the identification of transfected cells by fluorescence microscopy and flow cytometry.

Site-directed Mutagenesis—To assess whether the shRNA procedure was specific, wild-type (Cat or Mod) or mutant (Cat-mut or Mod-mut) forms of the rat catalytic or modulatory GCL subunit full-length cDNA were used. Cat-mut and Mod-mut were obtained by PCR site-directed mutagenesis using pIRES-Cat and pIRES-Mod, respectively, as template, followed by DpnI digestion (QuikChange XL, Stratagene). The selected PCR forward and reverse 50-nt oligonucleotides carried silent third codon base point mutations within the rat cDNA-corresponding Cat-d and Mod-c shRNA target sequences (5'-GAAAGAAGC-CACGTCAGTT-3' for Cat and 5'-GGCATCGCCACCTATCGAG-3' for Mod; with mutated nucleotides underlined).

Antibodies against the Catalytic and Modulatory GCL Subunits—Polyclonal antibodies against the catalytic and modulatory subunits of rat GCL were obtained by rabbit immunization with synthetic peptides (Mimotopes Pty. Ltd., Clayton, Victoria, Australia). The peptides were ¹⁶QRHADHVRRHGILQ²⁹ for the catalytic subunit and ²²⁹EALQESIP-DIEAQE²⁴² for the modulatory subunit. Rabbits were immunized with 100 ng of the peptides coupled to keyhole limpet hemocyanin according to the procedure described by Sambrook *et al.* (22). To improve the detection of the proteins, antibodies were purified from the antisera by affinity chromatography using columns of cyanogen bromide-activated Sepharose coupled to the peptides.

Neurons in Primary Culture—Cerebral cortex neurons in primary culture were prepared from fetal Wistar rats at 16 days of gestation (23). Dissociated cell suspensions were plated at a density of 2.5×10^5 cells/cm² in 6- or 12-well plates previously coated with poly-D-lysine (15 μ g/ml) in DMEM supplemented with 10% FCS. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and 95% air. Forty-eight hours after plating, the medium was replaced with DMEM supplemented with 5% horse serum, 20 mM D-glucose, and 10 μ M cytosine arabinoside to prevent non-neuronal proliferation. Neurons were transfected on day 5, when \sim 99% of the cells were microtubule-associated protein-2-positive.

Astrocytes in Primary Culture—Astrocytes in primary culture were prepared from the forebrains of neonatal rats (24 h old). Cell suspensions were seeded at a density of 1.2×10^5 cells/cm² in 175-cm² flasks in culture medium (DMEM supplemented with 10% FCS). Cells were incubated at 37 °C under a humidified 5% CO₂-containing atmosphere for 12 days, after which time $>$ 85% of the cells were astrocytes and the remaining proportion was microglia and progenitor cells as assessed by immunocytochemistry (23).

Neuron-Astrocyte Co-culture—Twenty-four h before the co-culture experiments, astrocytes were trypsinized and reseeded at 1.2×10^5 cells/cm² in Millicell-PCF cell culture inserts (4.2 cm² of effective membrane area, 0.4- μ m membrane pore size; Millipore Corp. Bedford, MA). To co-incubate astrocytes with neurons, astrocytes previously seeded in the cell culture inserts were placed on top of the neuronal cultures and bathed in DMEM (24). After 24 h of incubation, the inserts were removed, and the neurons were washed and used for the determination of either glutathione concentrations or apoptotic cell death. When necessary, neurons were transfected with the appropriate plasmid vectors 6 h before co-incubation.

Human Embryonic Kidney 293T (HEK293T) Cells—HEK293T cells were maintained in DMEM supplemented with 10% (v/v) FCS. Cells

Neuronal Glutathione shRNA Knockdown

were reseeded at 10^5 cells/cm² 1 day before transfections, after which FCS was reduced to 0.5% (v/v).

Cell Treatments—Cell transfections were performed with pSUPER-neo/GFP- or pIRES2-EGFP-derived plasmid constructions using Lipofectamine 2000 following the manufacturer's instructions. After 6 h, the medium was removed, and cells were further incubated for the indicated time periods in the presence of culture medium. To activate glutamate receptors, 24 h after transfections, neurons were incubated with 100 μ M glutamate (plus 10 μ M glycine) in buffered Hanks' solution (5.26 mM KCl, 0.43 mM KH₂H₂PO₄, 132.4 mM NaCl, 4.09 mM NaHCO₃, 0.33 mM Na₂HPO₄, 20 mM glucose, 2 mM CaCl₂, and 20 mM HEPES, pH 7.4). After 5 min, when glutamate receptors were activated (25), neurons were washed and further incubated in culture medium for the indicated time periods. To expose the transfected cells to nitric oxide, they were incubated in buffered Hanks' solution containing (Z)-1-[2-aminoethyl-N-(2-ammonioethyl)amino]diazene-1-ium-1,2-diolate (DETA/NO; Alexis Biochemicals) at 0.5 mM, a concentration seen to release continuously \sim 1.4 μ M nitric oxide at 37 °C, as measured using a nitric oxide-sensitive electrode (World Precision Instruments), for the indicated time periods. To ensure immediate exposure of the cells to nitric oxide, all DETA/NO-containing solutions were always preincubated in buffered Hanks' solution at 37 °C for 20 min before addition to the cells (23, 26).

Northern Blotting—Total RNA samples were purified (GenElute mammalian total RNA miniprep kit, Sigma) and electrophoresed (10 μ g of RNA/line) on a formaldehyde-containing 1% (w/v) agarose gel. After transfer to a GeneScreen Plus membrane (PerkinElmer Life Sciences) and cross-linking by ultraviolet irradiation, membranes were hybridized for 18 h at 65 °C in the presence of the appropriate random-primed [α -³²P]dCTP-radiolabeled cDNA probe (Amersham Biosciences, Buckinghamshire, UK) and exposed to Kodak XAR-5 film. We used the cDNA probes against rat Cat or Mod. A 0.7-kb cDNA fragment of the rat cyclophilin gene (generously donated by Dr. Dionisio Martín-Zanca, University of Salamanca) was used as a control of the amount of total RNA loaded onto each lane. Autoradiograms were scanned and quantified using NIH Image software. The scanned mRNA autoradiograms were expressed as a ratio *versus* cyclophilin mRNA.

Western Blotting—Cells were lysed for 20 min at 4 °C in buffer containing 1% Nonidet P-40, 5 mM EDTA, 2 mM EGTA, 20 mM Tris, pH 8.0, 137 mM NaCl, 10% glycerol, 100 μ M phenylmethylsulfonyl fluoride, 50 μ g/ml antipain, 50 μ g/ml pepstatin, 50 μ g/ml amastatin, 50 μ g/ml leupeptin, 50 μ g/ml bestatin, 1 mM *o*-vanadate, 50 mM NaF, and 50 μ g/ml soybean trypsin inhibitor. Extracts were centrifuged at 13,000 \times g for 20 min at 4 °C, and aliquots containing 100 μ g of protein from each sample, as determined extemporarily with the BCATM protein assay kit (Pierce) using albumin as a standard and the BenchMarkTM prestained protein ladder (Invitrogen), were subjected to SDS-PAGE on an 8% (catalytic subunit) or 12% (modulatory subunit) acrylamide gel (Mini-PROTEAN®, Bio-Rad). 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. GCL immunoblotting was performed using the anti-catalytic and anti-modulatory subunit antibodies that we raised (see above) at dilutions of 1:20 and 1:5, respectively. Anti-phosphofructokinase-1 antibody (1:500 dilution) was used as a loading control (23). Membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:10,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and immediately incubated with SuperSignal West Dura (Pierce) for 5 min before exposure to Kodak XAR-5 film for 1–3

min. Autoradiograms were scanned, quantified using NIH Image software, and expressed as a ratio *versus* phosphofructokinase-1.

GCL Activity—For the determination of GCL activity, we followed the method of Gegg *et al.* (27). In brief, 24 h after transfections, cells were washed, harvested by mild trypsinization, pelleted, and resuspended in isolation medium (320 mM sucrose, 10 mM Tris, and 1 mM EDTA, pH 7.4). Cell suspensions were freeze-thawed three times, and the lysates were filtered through 10-kDa molecular mass cutoff devices (Amicon, Inc., and Millipore Corp.) at 12,000 \times g for 15 min at 4 °C. The retained protein fraction was immediately assayed for GCL activity in 0.1 M Tris-HCl containing 0.15 mM KCl, 20 mM MgCl₂, 2 mM EDTA, 10 mM ATP, 10 mM L-cysteine, 40 mM L-glutamate, and 220 μ M acivicin, pH 8.2, at 37 °C for 20 min. The reaction was stopped by addition of ice-cold orthophosphoric acid (15 mM), and the reaction product was immediately used for γ -glutamylcysteine determination by high pressure liquid chromatography (Beckman Instruments) with electrochemical detection (ESA Biosciences, Inc., Chelmsford, MA) using a Techsphere ODS column (4.6 \times 250 mm, 5- μ m particle size) and 15 mM orthophosphoric acid as the mobile phase (27). An external standard of γ -glutamylcysteine (2.5–10 μ M) was used for the calculations. Preliminary experiments showed that, under these conditions, γ -glutamylcysteine synthesis was linear with the incubation time at least up to 20 min.

Glutathione Concentrations—Cells were washed with ice-cold phosphate-buffered saline and immediately scraped off the plastic with 1% (w/v) sulfosalicylic acid. Cell lysates were centrifuged at 13,000 \times g for 5 min at 4 °C, and the supernatants were used for the determination of total glutathione concentrations (GSH + 2 \times GSSG) on the same day using GSSG as a standard (0–50 μ M) as described previously (28–30).

Flow Cytometric Analysis of Apoptotic Cell Death—Allophycocyanin-conjugated annexin V and 7-aminoactinomycin D (7-AAD) (BD Biosciences) were used to quantitatively determine the percentage of apoptotic cells by flow cytometry. Cells were stained with allophycocyanin-conjugated annexin V and 7-AAD following the manufacturer's instructions and analyzed on a FACSCalibur flow cytometer (15-milliwatt argon ion laser tuned at 488 nm; CellQuest software, BD Biosciences). Both GFP-positive and GFP-negative cells were analyzed separately, and the allophycocyanin-conjugated annexin V-stained cells that were 7-AAD-negative were considered apoptotic (23).

Statistical Analysis—Measurements from individual cultures were always performed in triplicate. The results are expressed as the means \pm S.E. for three different culture preparations. Statistical analysis of the results was performed by one-way analysis of variance, followed by the least significant difference multiple range test. In all cases, $p < 0.05$ was considered significant.

RESULTS

Design, Expression, and Efficacy of shRNAs against the Catalytic and Modulatory GCL Subunits—To promote the specific silencing of GCL, five 19-nucleotide sequences were designed for each subunit according to a recently reported rational criterion for RNAi design for siRNA (20). These sequences were selected using the human GCL cDNA template within regions that were best conserved in rat (GenBankTM accession numbers NM_012815 (Cat) and NM_017305 (Mod)) and mouse (accession numbers NM_010295 (Cat) and NM_008129 (Mod)). Only those sequences that fulfilled at least six criteria were selected; these are shown in TABLE ONE. These sequences were subcloned into a GFP cDNA-coexpressing pSUPER-neo/GFP mammalian expression vector (19) to promote the expression of the corresponding shRNA molecules. The use of such an expression vector allows the specific and independent siRNA-mediated silencing of GCL subunits via the Dicer-triggered

TABLE ONE

Oligonucleotides (19 nucleotides) selected for siRNA against *Homo sapiens* GCL

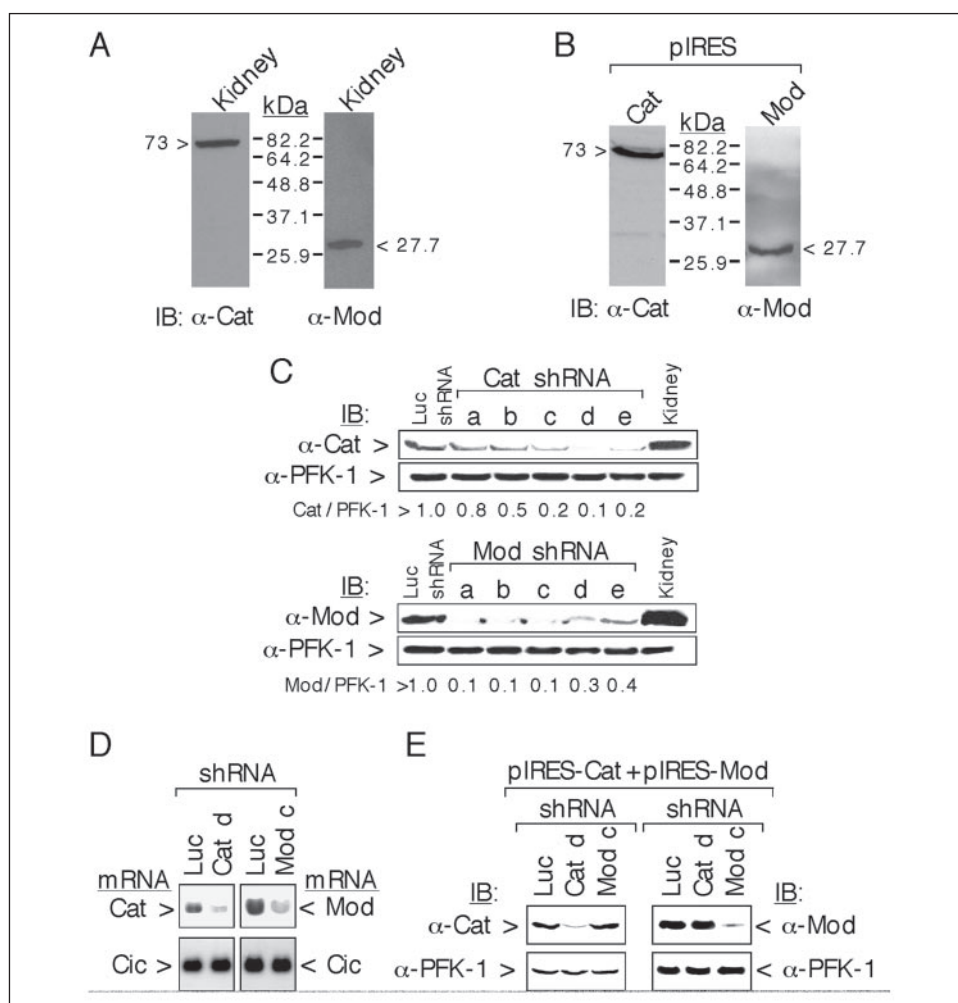
ORF, open reading frame.

Name	siRNA target sequence	Start	Criterion ^a							
			1	2	3	4	5	6	7	8
Catalytic subunit (accession no. NM_001498; 3717 bp; ORF region 389–2302)										
Cat-a	TCAGTACCTTAACAAGAAA	945	+	+	+	+	+	+	+	+
Cat-b	CTAAGCCGGATCATATTTA	1080	+	+	+	+	+	–	+	+
Cat-c	CCAGATACCTTTATGATCA	1173	+	+	+	+	+	+	+	+
Cat-d	GAAGGAGGCTACTTCTATA	772	+	+	–	+	+	+	+	+
Cat-e	GAGTGATCCTTTCCCTACAA	1737	+	+	+	+	–	+	+	+
Modulatory subunit (accession no. NM_002061; 1610 bp; ORF region 254–1078)										
Mod-a	ACAGCGAGGAGCTTCATGA	369	+	+	+	+	+	–	+	+
Mod-b	TTCACTCCTTGAGTTGCA	595	+	+	+	+	–	+	+	+
Mod-c	TGCTTCACCTCCTATTGAA	634	+	+	–	+	–	+	+	+
Mod-d	ACCAAATAGTAACCAAGTT	799	+	+	+	–	–	+	+	+
Mod-e	CCAAATAGTAACCAAGTTA	800	+	+	–	+	+	–	+	+

^a Satisfies (+) or not (–) the corresponding criteria reported previously (20) for the rational design of siRNA for RNA interference.

FIGURE 1. Expression and efficacy of shRNAs against the catalytic and modulatory GCL subunits.

A, Western blotting of rat kidney extracts revealed single bands at the appropriate molecular masses using antibodies raised against the catalytic (α -Cat) and modulatory (α -Mod) GCL subunits. **B**, immunoblot of HEK293T cells transfected with pIRES-derived plasmids encoding Cat and Mod cDNAs (pIRES-Cat and pIRES-Mod, respectively) afforded the corresponding bands by Western blotting. **C**, coexpression in HEK293T cells of pIRES-Cat with five different shRNAs targeted against the catalytic GCL subunit (upper panels) or pIRES-Mod with five different shRNAs targeted against the modulatory GCL subunit (lower panels) revealed different degrees of efficacy of the selected shRNAs in silencing the expression of the corresponding subunits compared with control luciferase (*Luc*) shRNA transfections. The shRNAs were expressed using the pSUPER-neo/GFP mammalian expression vector. In light of the different efficiencies, Cat-d and Mod-c shRNA sequences were used in ensuing experiments. Phosphofructokinase-1 (*PFK-1*) was used as a loading control. The bands were scanned and analyzed, and the Cat/phosphofructokinase-1 and Mod/phosphofructokinase-1 ratios (indicated under the corresponding condition) were used to estimate the efficiencies of the different shRNAs. **D**, transfection of HEK293T cells with Cat-d and Mod-c shRNAs decreased the abundance of the corresponding endogenous mRNAs as assessed by Northern blotting. Cyclophilin (*Cic*) was used as a loading control. **E**, transfection of HEK293T cells with shRNA against each GCL subunit did not affect the expression of the other subunit.



cleavage of shRNAs (18). To investigate the efficacy of the shRNA procedure, we obtained antibodies raised against the rat catalytic and modulatory GCL subunits. These antibodies proved to be highly specific, as revealed by the appearance of a single band of the expected size in rat kidney extracts by Western blotting (Fig. 1A). Because the endogenous GCL (Cat and Mod) protein levels were too low to be detectable by

Western blotting in cultured cells (data not shown), we estimated the efficacy of the selected shRNAs by assaying their ability to interfere with the respective expressed proteins. To accomplish this, we first obtained the full-length cDNAs for both the catalytic and modulatory GCL subunits by reverse transcription-PCR, and these were subcloned into the GFP cDNA-coexpressing pIRES mammalian expression vector. The

Neuronal Glutathione shRNA Knockdown

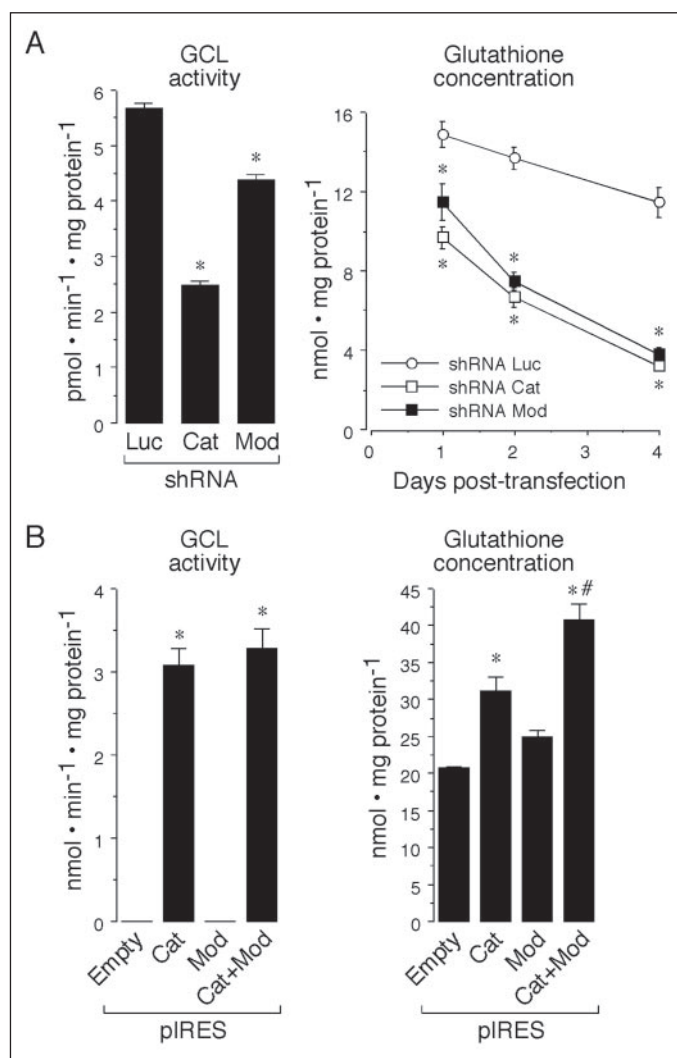


FIGURE 2. The catalytic and modulatory GCL subunits are essential for glutathione biosynthesis in intact cells. A, 24 h after transfection, specific disruption of the catalytic or modulatory subunit by shRNA in HEK293T cells significantly decreased GCL activity (left panel) and glutathione concentrations (right panel). B, overexpression of the catalytic (but not modulatory) GCL subunit alone was sufficient to significantly increase GCL activity (left panel) and glutathione concentrations (right panel). Concomitant overexpression of both subunits did not further enhance GCL activity (left panel), but synergistically increased glutathione concentrations (right panel). *, $p < 0.05$ compared with the corresponding control values (luciferase (Luc) in A and empty vector (Empty) in B); #, $p < 0.05$ compared with pIRES-Cat values.

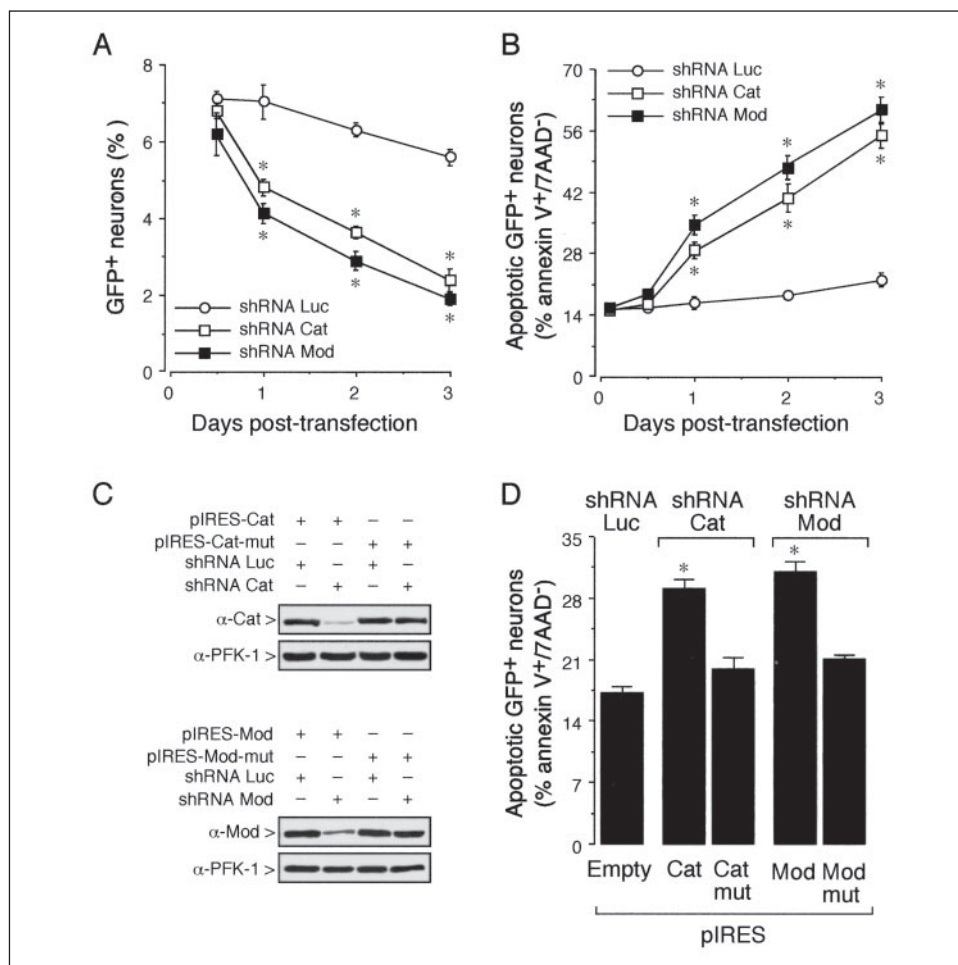
expression of these plasmids in HEK293T cells afforded proteins with the expected corresponding catalytic or modulatory GCL subunit sizes (Fig. 2B). The expression of five different shRNAs for each GCL subunit in HEK293T cells differentially disrupted the expression of the corresponding subunits (Fig. 1C) compared with control transfections, which were performed with shRNA against luciferase. In light of these results, we selected Cat-d and Mod-c shRNAs (Fig. 1C and TABLE ONE) because they were among the most effective shRNAs. The selected shRNAs (Cat-d and Mod-c) were then expressed in HEK293T cells, and their efficacy was assayed further by Northern blotting. As shown in Fig. 1D, Cat-d and Mod-c shRNAs efficiently decreased the endogenous levels of the catalytic and modulatory GCL subunit mRNAs, respectively. To corroborate further the specificity of this approach, we investigated whether the shRNA against each subunit affected the expression of the other subunit. As shown in Fig. 1E, Cat-d shRNA did not affect the protein levels of the modulatory subunit, and Mod-c shRNA did not affect the protein levels of the catalytic subunit. In view of the efficacy

and specificity of the selected shRNAs, all remaining experiments were performed using Cat-d and Mod-c shRNAs.

Both GCL Subunits Are Essential for Glutathione Biosynthesis in Intact Cells—The relative contribution of each GCL subunit to supporting GCL activity and glutathione concentrations in intact cells is still a controversial issue, with studies reporting either decreases (31) or no changes (32) in glutathione concentrations upon disruption of the modulatory GCL subunit using ribozyme or full-length antisense mRNA expression in pancreatic and hepatoblastoma cell cultures, respectively. Conversely, glutathione concentrations have been reported to be both maintained upon catalytic GCL subunit overexpression (10) and increased after catalytic or modulatory GCL subunit overexpression (33–35). Because these discrepancies do not allow the relative impact of each GCL subunit on neuronal survival to be clarified, we first investigated whether the independent disruption of GCL subunits by siRNA altered the enzyme activity and glutathione concentrations. To perform this, the shRNA targeted against the catalytic (Cat-d shRNA) or modulatory (Mod-c shRNA) GCL subunit was expressed in HEK293T cells, and enzyme activity and glutathione concentrations were measured in the cell extracts. Disruption of the catalytic subunit inhibited GCL activity by ~55%, whereas disruption of the modulatory subunit resulted in a modest but significant ~25% decrease in GCL activity (Fig. 2A, left panel). Likewise, total glutathione concentrations time-dependently decreased after transfections with Cat or Mod shRNA (Fig. 2A, right panel). Conversely, overexpression of the catalytic GCL subunit (pIRES-Cat) dramatically increased GCL activity (by ~500-fold). In contrast, overexpression of the modulatory GCL subunit (pIRES-Mod) did not affect GCL activity (Fig. 2B, left panel). Coexpression of both the catalytic and modulatory subunits did not result in further increases in GCL activity compared with overexpression of the catalytic subunit alone (Fig. 2B, left panel). Likewise, total glutathione concentrations increased (by ~1.4-fold) after overexpression of the catalytic GCL subunit, but were unmodified after overexpression of the modulatory subunit (Fig. 2B, right panel). Interestingly, the concomitant overexpression of both GCL subunits synergistically increased (by ~2-fold) glutathione concentrations compared with the overexpression of the catalytic subunit alone (Fig. 2B, right panel).

Both GCL Subunits Are Essential for the Survival of Primary Neurons—Neurons are especially vulnerable to oxidative and nitrosative stress (36–38), and the pharmacological inhibition of GCL activity with L-buthionine sulfoximine elicits apoptotic neuronal death (39). However, so far, there is no evidence indicating the relative contribution of each GCL subunit to neuronal survival and susceptibility to oxidative and nitrosative stress. Thus, we next studied whether the disruption of GCL activity, by specifically interfering with each GCL subunit, and the subsequent glutathione loss might be sufficient to trigger neurodegeneration. To address this issue, we transfected rat cortical neurons in primary culture with either Cat or Mod shRNA and then analyzed cell death. As shown in Fig. 3A, transfection with Cat or Mod shRNA, but not with luciferase shRNA (control), was associated with a progressive loss of GFP-positive neurons related to the incubation time. To determine apoptotic neuronal death, the proportion of annexin V-positive/7-AAD-negative neurons was assessed by flow cytometry within the GFP-positive subpopulation of neurons. As depicted in Fig. 3B, the number of GFP-positive apoptotic neurons increased progressively when transfected with Cat or Mod shRNA, but not with luciferase shRNA. To determine whether the apoptotic death of neurons was specifically due to GCL subunit silencing, we next constructed cDNAs coding for either the catalytic or modulatory GCL subunit carrying silent third codon base point mutations within the siRNA target

FIGURE 3. The catalytic and modulatory GCL subunits are essential for the survival of primary neurons. *A*, transfection of rat primary cortical neurons with the GFP cDNA-coexpressing pSUPER-neo/GFP vector coding for Cat or Mod shRNA time-dependently decreased the number of GFP-positive cells as assessed by flow cytometry. *B*, analysis of transfected (GFP-positive) neurons with annexin V and 7-AAD revealed that the shRNA-mediated disruption of Cat or Mod was sufficient to trigger a time-dependent apoptotic (annexin V-positive/7-AAD-negative) type of cell death. *C*, site-directed mutagenesis of pIRES-Cat or pIRES-Mod with silent point mutations at the third codon base within the shRNA target sequence (pIRES-Cat-mut or pIRES-Mod-mut, respectively) resulted in the expression of proteins that were refractory to the corresponding shRNA-mediated protein degradation. α -PFK-1, anti-phosphofruktokinase-1 antibody. *D*, expression of the catalytic or modulatory GCL subunit cDNA carrying silent mutations within the shRNA target sequence (pIRES-Cat-mut or pIRES-Mod-mut, respectively) prevented Cat or Mod shRNA-mediated apoptotic neuronal death. *, $p < 0.05$ compared with the corresponding control values (luciferase (*Luc*) in *A* and *B* and empty vector (*Empty*) in *D*).



sequence (Cat-mut or Mod-mut), thus expressing mRNAs refractory to the action of the shRNAs but having functional translational products. As shown in Fig. 3C, the expression of Cat shRNA promoted protein disruption of the wild-type (but not mutant) GCL subunits. Moreover, expression of the mutant forms of both GCL subunits fully prevented the apoptotic neuronal death triggered by the corresponding Cat or Mod shRNA (Fig. 3D).

Neuronal Death by GCL Knockdown Is Prevented by Glutathione Ethyl Ester or γ -Glutamylcysteine, but Not by Co-incubation with Astrocytes—Previous studies have reported that glutathione concentrations (36, 40) and GCL expression (41–43) in neurons increase after co-incubation with astrocytes, and these factors may contribute to the beneficial role of astrocytes in protection against neuronal oxidative damage. In fact, astrocytes supply neurons with not only precursors for the biosynthesis of glutathione (40, 44), but possibly also other antioxidants such as vitamin E and ascorbate (45). In view of these observations and with the aim of determining the putative effectiveness of GCL disruption *in vivo*, we investigated whether the presence of astrocytes in co-culture altered neuronal survival upon siRNA-mediated disruption of GCL activity. First, we observed that the site of glutathione inhibition was exclusively exerted at the step catalyzed by GCL. Thus, both the GCL product, γ -glutamylcysteine, and the membrane-permeable glutathione analog glutathione ethyl ester increased neuronal glutathione concentrations (Fig. 4A) and prevented neuronal apoptosis triggered by specific shRNA-mediated GCL silencing (Fig. 4B). The presence of astrocytes in co-culture increased glutathione concentrations (Fig. 5A) in normal untransfected neurons, confirming previous results (36, 40).

However, the presence of astrocytes in co-culture did not rescue the apoptotic neuronal death caused by shRNA-mediated catalytic or modulatory GCL subunit blockade (Fig. 5B).

Modulation of Neuronal Vulnerability to Glutamate- and Nitric Oxide-mediated Toxicity through shRNA-mediated Inhibition or cDNA Overexpression of GCL Subunits—A large body of evidence suggests that glutathione deficiency enhances neuronal susceptibility to oxidative and nitrosative stress (24, 42, 43, 46), reflecting the relevance of glutathione loss for neurodegenerative diseases. However, it should be noted that this idea was obtained only after the inhibition of pharmacological GCL activity, hence questioning any high degree of confidence in the specificity of the effect. Here, we addressed the issue of whether single GCL subunit silencing or overexpression would be sufficient to modulate neuronal vulnerability to the activation of endogenous glutamate receptors or to nitric oxide. Neurons pretreated with the shRNAs targeted to GCL subunits for 24 h were further incubated with glutamate (100 μ M for 5 min), a model that we have previously shown to trigger activation of glutamate receptors in these cells (particularly N-methyl-D-aspartate receptors (25)) and nitric oxide production and neuronal death (47) via mitochondrial dysfunction (25). Glutamate treatment was sufficient to enhance apoptotic cell death within the GFP-negative neurons, *i.e.* neurons not expressing the shRNAs (Fig. 6A, left panel). Furthermore, the time-dependent increase in apoptotic neuronal death caused by glutamate treatment was synergistic with the shRNA-mediated silencing of GCL subunits as judged by the proportion of the apoptotic shRNA-expressing GFP-positive subpopulation of neurons (Fig. 6A, right panel). It should be noted that, within this GFP-

Neuronal Glutathione shRNA Knockdown

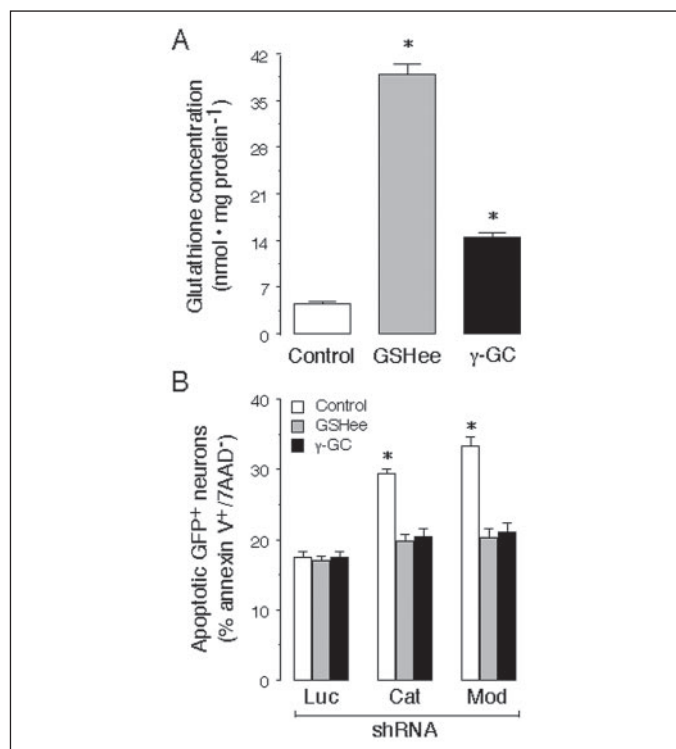


FIGURE 4. Neuronal death by GCL knockdown is prevented by glutathione ethyl ester or γ -glutamylcysteine. *A*, incubation of rat primary cortical neurons with glutathione ethyl ester (GSHee; 5 mM) or γ -glutamylcysteine (γ -GC; 5 mM) for 18 h significantly increased intracellular glutathione concentrations. *B*, incubation of neurons with glutathione ethyl ester or γ -glutamylcysteine fully prevented subsequent Cat or Mod shRNA-mediated apoptotic neuronal death. *, $p < 0.05$ compared with the corresponding control values (luciferase (Luc) in *B*).

positive subpopulation, the considerable degree of cell death observed in control (glutamate-untreated) shRNA Cat or Mod neurons (Fig. 6*A*, right panel) was due to the silencing effect of these subunits themselves, as reported above (Fig. 3*B*). Similarly, treatment with nitric oxide enhanced apoptotic cell death within the shRNA-free GFP-negative neurons (Fig. 6*B*, left panel). Furthermore, the neuronal death caused by nitric oxide treatment was synergistic with the shRNA-mediated silencing of each GCL subunit (Fig. 6*B*, right panel). Finally, apoptotic neuronal death due to glutamate (Fig. 7*A*) or nitric oxide (Fig. 7*B*) treatment was prevented by overexpression of the catalytic (but not modulatory) subunit of GCL.

DISCUSSION

Here, we have shown that independent inhibition of either the catalytic or modulatory GCL subunit by the shRNA strategy elicits spontaneous neurodegeneration in primary cortical neurons. To our knowledge, this is the first report demonstrating the absolute requirement of both GCL subunits for neuronal survival. Knockdown of GCL subunits leading to neuronal death was specific and selective because (i) the expression of site-directed mutant GCL subunit cDNA constructs in the siRNA sequence afforded cells refractory to shRNA-mediated protein degradation and apoptotic death; (ii) the exogenous addition of products beyond the GCL step, such as γ -glutamylcysteine and glutathione ethyl ester, fully prevented shRNA-mediated neuronal death; and (iii) the presence of astrocytes (which supply precursors, but not products beyond the GCL step (40, 44)) did not prevent shRNA-mediated neuronal death. Together, these results suggest that shRNA against either GCL subunit would be a suitable model for the intrinsic deficiency of glutathione, leading to oxidative stress in cultured neurons.

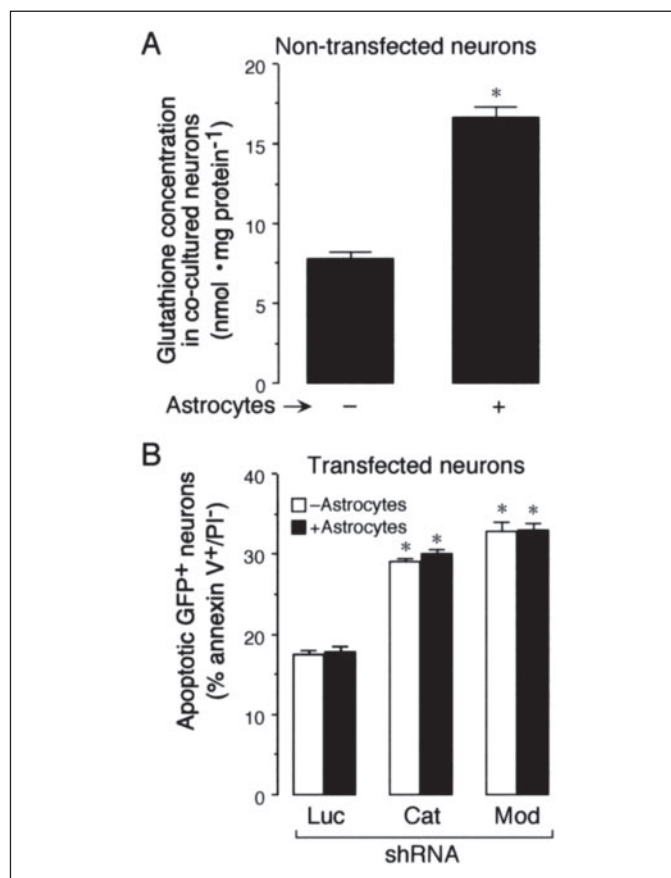
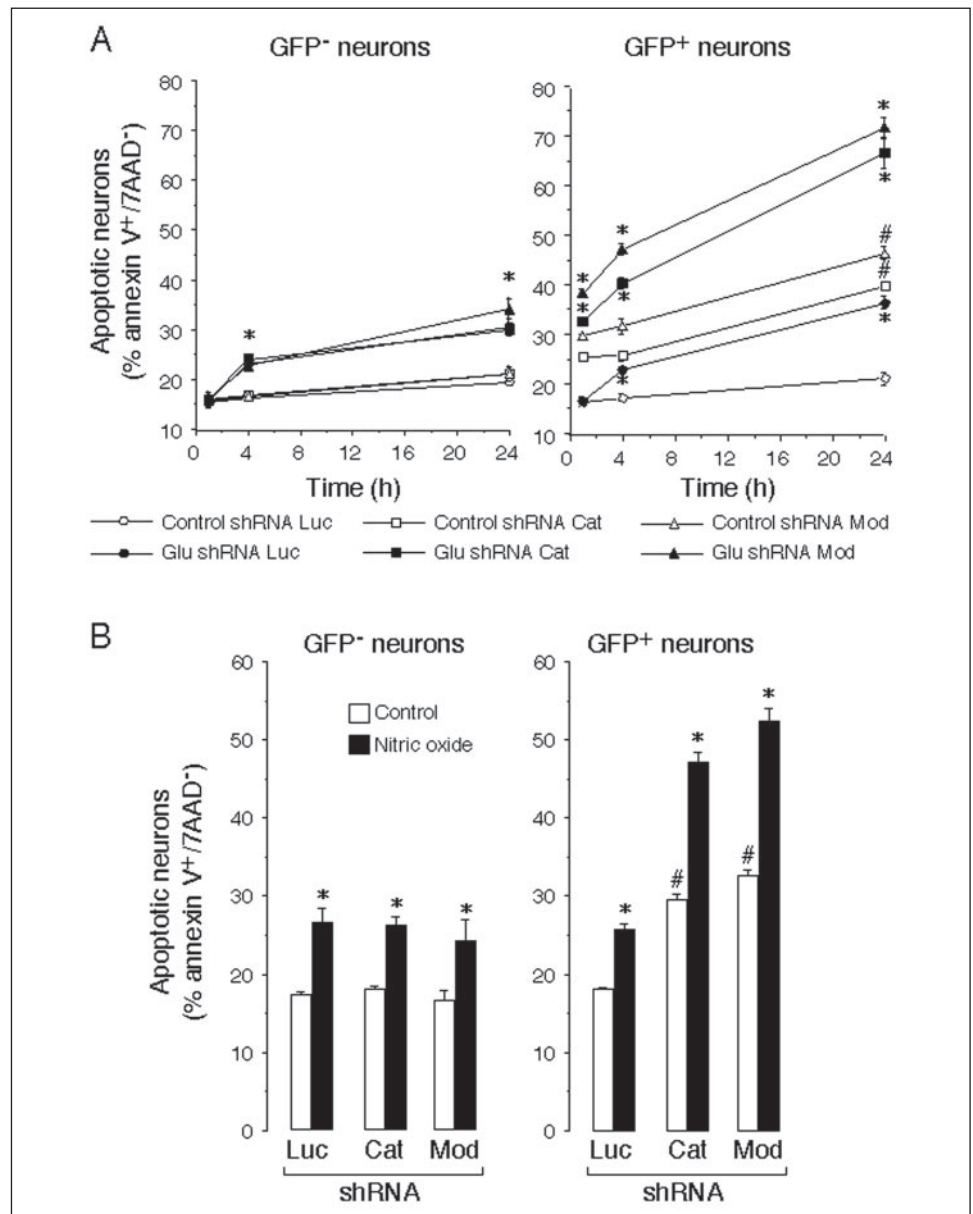


FIGURE 5. Neuronal death by GCL knockdown is not prevented by co-incubation with astrocytes. *A*, incubation of rat primary cortical neurons with cell co-culture inserts containing astrocytes (+) for 18 h significantly increased intracellular glutathione concentrations in the neurons compared with inserts not containing astrocytes (-). *B*, incubation of Cat or Mod shRNA-transfected neurons with astrocytes in co-culture did not prevent subsequent shRNA-mediated apoptotic neuronal death. *, $p < 0.05$ compared with the corresponding control values (- astrocytes in *A* and luciferase (Luc) in *B*).

Our data showing that the specific disruption of the catalytic GCL subunit was sufficient to cause decreases in enzyme activity and glutathione concentrations confirm previous work (11). In contrast, the decrease in GCL activity and glutathione concentrations observed after inhibition of the modulatory GCL subunit either agrees (31) or disagrees (32) with previous studies performed in different models. The modulatory subunit essentially serves to increase the affinity of the catalytic GCL subunit for glutamate and to reduce feedback inhibition by glutathione (9, 13). This is supported by our data, which show that, although its overexpression alone did not induce any changes in GCL activity, glutathione concentrations, or neuronal survival, its disruption did elicit decreases in glutathione concentrations. This confirms the critical role of the modulatory subunit in regulating GCL activity in intact cells (9, 13) and in dictating neuronal survival (this work). Moreover, disruption of this subunit alone enhanced (as did the catalytic subunit) neuronal susceptibility to nitric oxide, formed either endogenously by glutamate receptor activation or exogenously by addition of a nitric oxide donor to the cultured primary neurons. In this context, we have previously reported that the catalytic GCL subunit can be induced by nitric oxide in astrocytes, but not in neurons (42). Together, these data confirm the idea that neurons are especially vulnerable to nitric oxide-mediated mitochondrial damage and neurotoxicity (25, 26, 36) and that glutathione plays an important role in neuronal survival (24). A recently reported work performed in differentiated PC12 cells shows that endogenous nitric oxide inhibits mitochondrial complex I activity after con-

FIGURE 6. GCL knockdown enhances neuronal vulnerability to glutamate- and nitric oxide-mediated toxicity. Rat primary cortical neurons were transfected with Cat or Mod shRNA; and 24 h after transfection, cells were treated with glutamate or nitric oxide as indicated. *A*, incubation of neurons with glutamate (100 μ M for 5 min) time-dependently enhanced the apoptotic death of GFP-negative neurons (*left panel*), an effect that was synergistic with the shRNA-mediated silencing of GCL subunits as judged by the analysis of GFP-positive apoptotic neurons (*right panel*). *B*, incubation of neurons with nitric oxide ($\sim 1.4 \mu$ M, released from DETA/NO) enhanced the apoptotic death of GFP-negative neurons (*left panel*), an effect that was synergistic with the shRNA-mediated silencing of GCL subunits as judged by the analysis of GFP-positive apoptotic neurons (*right panel*). *, $p < 0.05$ compared with the corresponding control values (not treated with glutamate or nitric oxide); #, $p < 0.05$ compared with luciferase (*Luc*) shRNA.



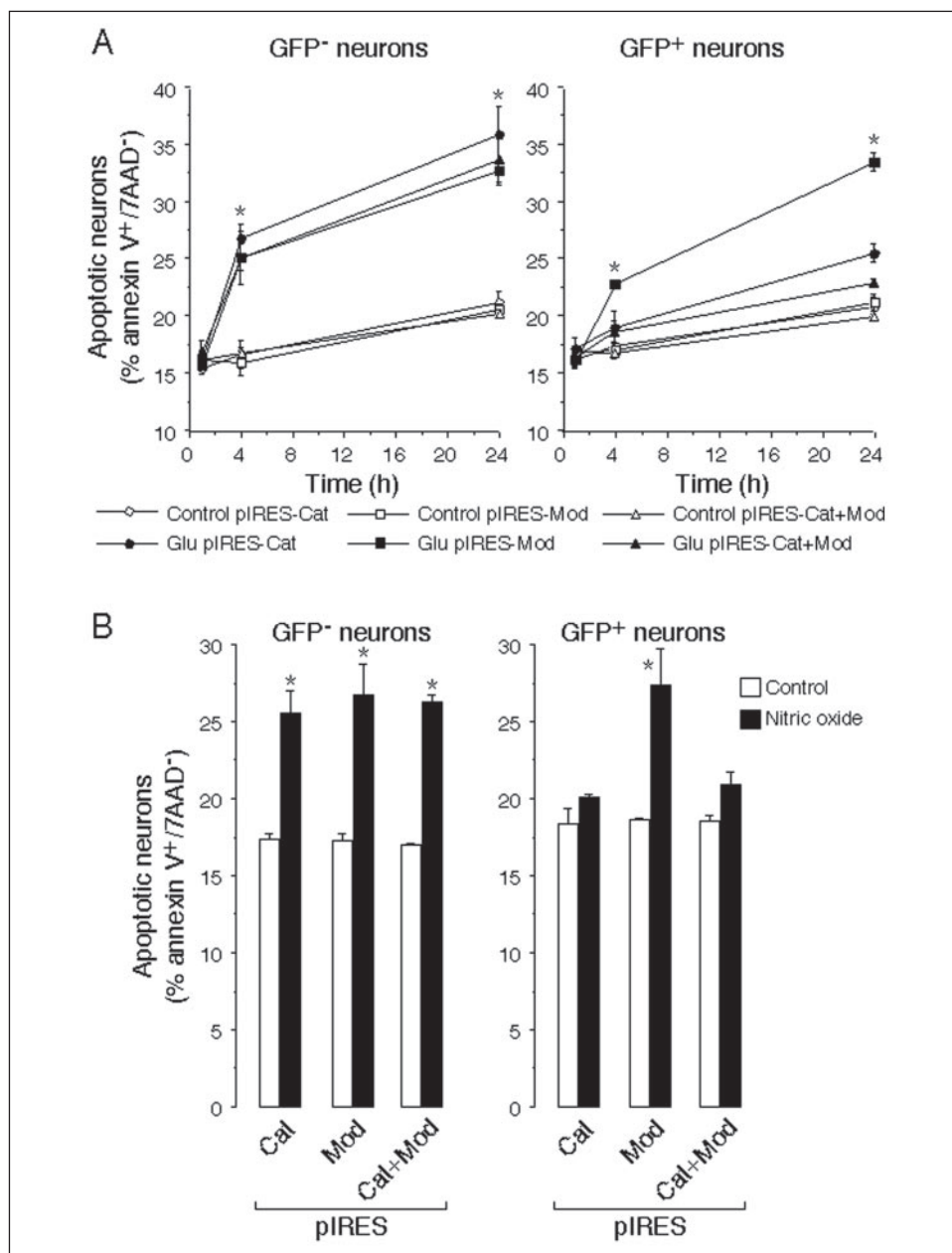
comitant depletion of both the catalytic and modulatory GCL subunits using an RNA antisense strategy (17). Here, using the more specific siRNA strategy (18) in primary cells, we have further shown that independent disruption of GCL subunits is sufficient to elicit neuronal death. These results represent the first report indicating that each GCL subunit is essential for the survival of neurons. Whether neuronal death might be mediated by complex I inhibition (17) is a tempting speculation that we were unable to explore due to the low transfection efficiencies obtained ($\sim 7\%$), but it is certainly an explanation that merits further investigation.

Previous studies performed *in vivo* have shown that targeted disruption of the catalytic GCL subunit gene in mice is lethal after embryonic day 8 (11, 12). This is supported by our work showing that the shRNA-mediated catalytic GCL subunit inhibition was sufficient to trigger apoptotic neuronal death. Moreover, the inhibition of this subunit enhanced the vulnerability of neurons to glutamate receptor activation and nitric oxide, whereas its overexpression was markedly protective. Together, these data unambiguously confirm the widely accepted

notion that the catalytic subunit is an essential protein for glutathione biosynthesis and cellular viability.

In contrast, modulatory GCL subunit-targeted disruption has been reported to yield viable and fertile mice showing mild and chronic glutathione deficiency in a number of tissues (including liver, kidney, and pancreas), erythrocytes, and blood (13); unfortunately, brain tissue was not investigated. In this context and in light of the results demonstrating the good viability of animals upon modulatory GCL subunit-targeted disruption, it is intriguing that we found this to be incompatible with the survival of primary neurons. Some redundancy in the function of the modulatory subunit must exist *in vivo* (13) that is not evident in the *in vitro* culture system used in our work. Although this would help to explain the apparent discrepancy, its confirmation will require a full examination of the brain cells of modulatory GCL subunit knockout mice. In this context, it should be noted that, in the more *in vivo*-like microenvironment (such as astrocytes in co-culture), neuronal glutathione is enhanced (see Refs. 24 and 40). However, the blockade of glutathione biosynthesis in neurons by the modulatory or catalytic GCL sub-

FIGURE 7. GCL overexpression reduces neuronal vulnerability to glutamate- and nitric oxide-mediated toxicity. Rat primary cortical neurons were transfected with pIRES-Cat or pIRES-Mod; and 24 h after transfection, cells were treated with glutamate or nitric oxide as indicated. *A*, incubation of neurons with glutamate (100 μ M for 5 min) time-dependently enhanced the apoptotic death of GFP-negative neurons (*left panel*), an effect that was fully prevented by pIRES-Cat or pIRES-Cat + pIRES-Mod cotransfection, but not by pIRES Mod as judged by the analysis of GFP-positive apoptotic neurons (*right panel*). *B*, incubation of neurons with nitric oxide ($\sim 1.4 \mu$ M, released from DETA/NO) enhanced the apoptotic death of GFP-negative neurons (*left panel*), an effect that was fully prevented by pIRES-Cat or pIRES-Cat + pIRES-Mod cotransfection, but not by pIRES Mod as judged by the analysis of GFP-positive apoptotic neurons (*right panel*). *, $p < 0.05$ compared with the corresponding control values (not treated with glutamate or nitric oxide).



unit shRNA still promoted neuronal death despite the presence of astrocytes. This result was expected because astrocytes do not provide neurons with glutathione itself, but with its precursors cysteine and glycine (in the form of cysteinylglycine) (40). In fact, exogenous addition of the plasma membrane-permeable form of glutathione (glutathione ethyl ester) or the GCL product (γ -glutamylcysteine) fully prevented the neuronal death caused by GCL subunit shRNA. These data reinforce the notion that specific targeting of the modulatory as well as catalytic GCL subunits in neurons would be essential for their survival *in vivo*, at least during the late stages of differentiation.

In conclusion, we have demonstrated that both GCL subunits are essential for the survival of neurons. Furthermore, we have provided a powerful and specific tool that may be useful for understanding the mechanisms leading to neurodegeneration due to spontaneous oxidative stress. In this sense, it is interesting to note that the deleterious effects of free radicals occur during adulthood, suggesting that oxidative stress is a critical factor in triggering aging (6, 48) as well as neurode-

generation (2, 4, 5). If so, our model of the specific and independent disruption of GCL by the shRNA strategy might help to clarify whether the modulation of glutathione biosynthesis *in vivo* is an essential factor dictating neuronal death in neurodegenerative conditions such as Parkinson disease.

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