Supporting Information

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SI Materials and Methods

Reagents. DMEM, FBS, and calf serum were purchased from Invitrogen. Epoxomicin and MG132 were purchased from Sigma. Antibody for Nrf1 was previously described (1). Antibodies for proteasome subunits PSMα1–α7, β1, β2, β5, Rpt2, and Rpt5 were from Enzo Life Sciences. Antibody for β-actin (A1978) was from Sigma. Antibodies against cleaved-caspase 3, cleaved-caspase 9, and ubiquitin were from Cell Signaling. Anti-NeuN was from R&D Systems. Proteasome substrates Suc-Leu-Leu-Val-Try-7-amino-4-methylcoumarin, Boc-Leu-Arg-Arg-7-amino-4 methyl-coumarin, and Asp-7-amino-4-methylcoumarin were from Enzo Life Sciences. Biotinylated anti-rabbit IgG and 3,3′ diaminobenzidine (DAB) substrate kit are from Vector Laboratories. Alexa 488-conjugated goat anti-mouse IgG and Alexa 568 goat anti-rabbit IgG were purchased from Invitrogen.

Histology and Immunohistochemistry. Mice were transcardially perfused with 4% paraformaldehyde (PFA) fixative. Brains were dissected and further fixed in 4% PFA overnight at 4 °C, cryoprotected in 15–30% sucrose overnight, and sectioned in the coronal plane using a cryostat. Brains were cut at 40 μm, free-floated in PBS, and mounted on Superfrost glass slides. Sections were stained with primary antibody in blocking solution overnight at 4 °C, rinsed in Tris-buffered saline Tween 20 buffer, and then incubated for 1 h with biotinylated anti-rabbit IgG (1:300). Visualization of the product was performed using DAB substrate kit. For immunofluorescence assay, after incubation with primary antibody overnight at 4 °C, sections were then incubated with Alexa 488-conjugated goat anti-mouse IgG or Alexa 568 goat anti-rabbit IgG secondary antibodies (1:300). Tissue sections were examined using a Nikon epifluorescent microscope equipped with a CCD camera.

Stereology. Stereology was performed as previously described (2). Briefly, the total numbers of neurons were counted in the sensorimotor cortex at the level of anterior hippocampus (between bregma −1.28 and −1.64). Each section was outlined at low power (4×), and the numbers of NeuN-positive cells were counted at high power (100× oil; NA 1.4) using a 50×50-μm counting frame using a computer-assisted image analysis system consisting of an Olympus BX-51 microscope equipped with a XYZ computer-controlled motorized stage and Stereo Investigator software (MBF Bioscience).

In Vitro Measurement of Proteasomal Function. Freshly dissected brains were homogenized (10% wt/vol) in ice-cold buffer [10% glycerol, 25 mM Tris-HCL (pH 7.4), 10 mM MgCl$_2$, 4 mM ATP, and 1 mM DTT] using a polytron and centrifuged at $12,000 \times g$ for 15 min at 4 °C to remove debris. Protein concentrations were determined by the Bradford assay with BSA as protein standard. Proteasomal enzymatic activity was determined by fluorometric assays using Suc-Leu-Leu-Val-Try-7-amino-4-methylcoumarin (Suc-LLVY-AMC), Boc-Leu-Arg-Arg-7-amino-4-methylcoumarin (Boc-LRR-AMC), or Ac-Nle-Pro-Nle-Asp-7-amino-4-methylcoumarin (Ac-nLPnLD-AMC) as substrates. Reactions were initiated by adding 50 μM substrate followed by incubation at 37 °C for 30 min. Release of fluorogenic AMC was monitored at 360-nm excitation and 460-nm emission using a Molecular Devices fluorometric plate reader. Fluorescence units were converted to AMC concentration by using standard curves generated from free AMC. Rates were expressed as μmol of AMC per second per gram brain tissue protein. Epoxomicin, a proteasome-specific inhibitor, was

used to ensure specificity of the assays. In a separate assay, a 15-min preincubation of samples with epoxomicin at room temperature was performed before adding fluorogenic substrates. For in-gel assays, equal amounts of brain lysates from 1-mo-old control and Nrf1BKO mice were separated by native gel electrophoresis and assayed for 26S and 20S proteasome complexes by overlying gels with substrates. Brain extracts were electrophoresed on 4.5% nondenaturing polyacrylamide gel with 2.5% stacking gel, 90 V for 4 h at 4 °C. After electrophoresis, gels were incubated with proteasome substrates (Suc-LLVY-amc, Boc-LRR-amc, or Ac-nLPnLD-amc) for 30 min at 37 °C. The 20S core particle was detected by incubating the gel in the presence of 0.02% SDS to activate the 20S core. Active proteasome bands were then visualized by exposure to UV light at 360 nM, using Fujifilm Global (LAS-4000). Band intensity was quantified by using ImageReader software (Fujifilm).

TUNEL Analysis. Slides were air dried and fixed with freshly made 4% PFA for 1 h at room temperature. After rinsing with PBS, cells were incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. Cells were then labeled with fluorescein dUTP to free 3′-OH DNA ends using terminal deoxynucleotidyl transferase (TdT) at 37 °C in a humidified chamber for 60 min (In Situ Cell Death Detection Kit; Roche). For positive control, cells were first treated with DNase I to induce DNA strand breaks, before TUNEL labeling. For negative control, cells were incubated in TUNEL reaction mixture without TdT. After labeling, samples were analyzed using a Nikon epifluorescent microscope equipped with a CCD camera.

GSH and GSSG Assays. Dissected brains were homogenized on ice with 10 volumes of PBS to measure reduced glutathione (GSH) level and with 10 volumes of PBS containing 10 mM 1-methyl-2-vinylpyridinium trifluoromethanesulfonate to measure oxidized glutathione (GSSG) level. Protein samples were cleared by centrifugation at $13,000 \times g$ for 5 min at 4 °C, and supernatants were collected and aliquots stored at −70 °C until assay. GSH and GSSG levels were measured by enzymatic assays using a kit from Oxis International. Detection of reaction products was monitored every 10 s over a period using a microplate reader (Versamax; Molecular Devices) set at 412 nm. Levels were calculated from standard curves of GSH and GSSG.

Flow Cytometric Measurements of Cellular Redox Status. Cells were trypsinized and resuspended in PBS containing 0.5% BSA. To measure intracellular reactive oxygen species level, cell suspensions were incubated with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) for 30 min at 37 °C and washed with PBS. Oxidative conversion of DCFHDA to its fluorescent product, 2′,7′-dichlorofluorescein (DCF), was then assessed by the amount of fluorescent signal in live cells by flow cytometry. Propidium iodide (1 μg/mL) was added before analysis to gate out dead cells in the analysis.

Transcriptional Profiling. Total RNA from the frontal cortices of 3- to 4-wk-old Nrf1BKO ($n = 3$) and matched controls ($n = 3$) was processed on oligonucleotide arrays (Affymetrix mouse exon array 1.0 ST) according to the manufacturer's protocol and analyzed using Partek Genomics Suite 6.5. The exon array cel files were quantile normalized, and an ANOVA was run to compare expression of Nrf1BKO and matched controls using the 28,874 summarized transcript values. This list of selected genes was queried for enrichment of biological functions using Ingenuity

Pathway Analysis v8.7 software to identify biological processes that might be affected in the Nrf1 brain knockouts. All nominally significant ($P < 0.05$) up- or down-regulated transcripts with fold changes above 1.1 or below −1.1 were used as the input variables for the dataset to query the Ingenuity Pathways Analysis (IPA) software canonical pathway analysis, and overrepresentation of up- and down-regulated genes were run analyzed separately. Transcripts were mapped to corresponding gene objects in the Ingenuity Pathways Knowledge Base. Each network or pathway was set to have a maximum of 35 focus genes, and IPA identified those pathways that were most significant relative to the input dataset. The significance of the association between the dataset and the canonical pathway was determined according to the Benjamini-Hochberg step-down false discovery rate (FDR) calculated with the Fisher's exact test by calculating the probability that the association between the genes in the dataset and the canonical pathway is due to chance alone.

Primary Neuron Cultures. Cortical neurons from embryos were isolated and cultured. Briefly, brains were dissected from embryonic day 14–15 embryos, and meninges were removed in ice-cold calcium- and magnesium-free Hanks balanced salt solution (CMF-HBSS) media. Only the cortical area of the brain was used for culture. Isolated cortices were minced and digested with 0.25% trypsin-EDTA in a 37 °C water bath for 30 min. Single cells were made by triturating the tissue with P-1000 pipette tips, followed by filtering through a 70-μm cell strainer. Neurons were cultured on poly-L-lysine–coated plates in Neurobasal medium supplemented with 2% B27, 1% penicillin:streptomycin solution, and 0.5% L-glutamine. Cultures were replaced with 50% fresh media every 3 d.

RNA Isolation and Quantitative RT-PCR. RNA was extracted using UltraSpec RNA (Biotecx). cDNAs were synthesized from 10 μg total RNA in 20-μL reactions containing 1× RT buffer, 1 mM dNTPs, 0.3 μg random hexamer, 40 U of RNase inhibitor, and 250 U of Moloney murine leukemia virus reverse transcriptase. Reverse transcription reactions were incubated at 72 °C for 5 min and then 25 °C for 10 min, followed by 42 °C for 60 min. Aliquots of cDNA were amplified in a Step One Plus PCR machine (Applied Biosystems) using FastStart SyBr Green reagent (Roche) in duplicates in 20-μL reaction volumes. Sequences of the PCR primers are listed in Table S1. PCR cycling conditions consist of 95 °C for 15 min and 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 68 °C for 45 s. Expression levels were calculated relative to 18s rRNA levels as endogenous controls. Relative expression was calculated as $2^{(\text{Ct test gene} - \text{Ct 18s})}$.

Western Analysis. Protein lysates were made using Nonidet P-40 lysis buffer and electrophoresed on SDS-polyacrylamide gels. Proteins were subsequently transferred onto nitrocellulose membranes and blocked in 5% milk at room temperature for 1 h. Primary antibody incubation was done overnight at 4 °C followed by incubation with a horseradish peroxidase-conjugated secondary antibody. Proteins were visualized using a chemiluminescent detection system (Pierce Biotechnology).

Lentivirus Production and Infection. Virus production was done according to Tiscornia et al. (3). HEK293 cells were cotransfected with LKO.1 lentiviral, Delta VPR 8.9, and VSV-G plasmids using Lipofectamine 2000 (Invitrogen). Virus-containing supernatant was collected 48 h after transfection and filtered through a 0.45-μm

cellulose acetate filter (Millipore). Infections of cells were carried out in the presence of 10 μg/mL polybrene and 10 mM Hepes. After transduction, cells were selected with puromycin for stable clones.

Colony-Forming Assay. Cells (4×10^5) were seeded in six-well plates overnight and then treated with 15 μM and 30 μM of MG132 or with DMSO at 37 °C. After 20 h of drug treatment, cells were trypsinized and reseeded in new six-well plates at 1:20 and 1:10 dilution in fresh culture medium without drug. Cells were grown until colonies were visible (after 3 d), counted, and the percentage of colonies relative to DMSO-treated controls was calculated.

Plasmids. The luciferase reporter driven by the PsmB6 promoter was generated by PCR amplification of mouse genomic DNA using the forward and reverse primers GGCCGCCATCTTCCTCTGCTA and TGATGGCTCATCGCCATCCAT, respectively, and cloned into the NheI and XhoI sites of pGL3-Basic (Promega). The Nrf1 expression vectors were previously described (1). Ub-G76V-GFP and Ub-M-GFP plasmids were purchased from Addgene. To generate Ub-G76V-RFP and Ub-M-RFP plasmids, Ub-G76V and Ub-M were digested in EcoRI and BamHI and cloned into EcoRI and BamH1 sites of the pDsRed2-N1 from Clontech.

Transient Transfection and Luciferase Assays. HEK293 and MEF cells were grown in DMEM supplemented with 10% FCS and 100 μg/mL of each streptomycin and 100 U/mL penicillin at 37 °C in a humidified, 5% CO_2 atmosphere. Cells were seeded into 24-well plates at least 12 h before transfection. Cells were transfected using Lipofectamine 2000 reagent according to the manufacturer's protocol. After 48 h, cellular extracts were prepared, and luciferase activities were measured with the Dual Luciferase Reporter Assay Kit (Promega) using a TD-20/20 luminometer (Turner Designs). Firefly luciferase values were normalized to Renilla luciferase control. Transfection experiments were repeated at least three times, and data points were calculated as the mean of the results (three wells per experiment).

Chromatin Immunoprecipitation Assay. We used a kit from Upstate Biotechnology. Briefly, cells were fixed with 1% fomaldehyde at room temperature, and the reaction was stopped by glycine. Cells were washed with PBS followed by lysis in SDS buffer. Cells were then sonicated, and the supernatant was precleared with protein-A beads and sheared herring sperm DNA. The supernatant was then incubated with Nrf1-specific rabbit polyclonal or unrelated rabbit polyclonal as a control at 4 °C overnight. The DNA/protein complexes were then washed with low-salt wash buffer, followed by high-salt wash and then TE buffer. DNA was eluted with elution buffer at room temperature. Cross-links were reversed by heating at 65 °C for 5 h in 5 M NaCl. Samples were then treated with RNase and proteinase K, and DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Relative amounts of DNA in the complex were quantified by a real-time PCR method using primers flanking the gene promoter. Amplification specificity was checked using melting curve and agarose gel electrophoresis. NADPH oxidoreductase-1 and lactate dehydrogenase were used as positive and negative controls, respectively.

Statistical Analysis. Data were expressed as means ± SEM. For statistical comparison, Student t test or one-way ANOVA were used. P values of <0.05 were considered statistically significant.

1. Wang W, Chan JY (2006) Nrf1 is targeted to the endoplasmic reticulum membrane by an N-terminal transmembrane domain. Inhibition of nuclear translocation and trans-acting function. *J Biol Chem* 281:19676–19687.
2. Zhang J, et al. (2007) Essential function of HIPK2 in TGFbeta-dependent survival of midbrain dopamine neurons. *Nat Neurosci* 10:77–86.
3. Tiscornia G, Singer O, Ikawa M, Verma IM (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci USA* 100:1844–1848.

Fig. S1. Nrf1 is highly expressed in mouse brain and neurons. (*A*) In situ hybridization of a representative coronal section through the mouse brain showing Nrf1 expression in multiple brain regions. Note intense hybridization of Nrf1 antisense probe in the cortex (C), dentate gyrus (DG), CA1 and CA3 fields of the hippocampus, and choroid plexus (ChP). *Right:* Coronal section showing lack of hybridization using Nrf1 sense probe. (*B*) Immunofluorescent staining of Nrf1 (green) and Neu-N (red) in the cortex. Merged image shows costaining of Nrf1 and NeuN. (*C*) Immunofluorescent staining of Nrf1 (green) and Neu-N (red) in mouse primary cortical neurons. Merged image shows Nrf1 expression in neurons.

Fig. S2. Conditional Nrf1 knockout in the brain. (*A*) Diagram of the floxed *nrf1* locus showing *lox*P elements (filled circles) flanking the terminal exon of the gene and the predicted product of Cre-mediated recombination. Primers used to detect nonrecombined and recombined floxed *nrf1* alleles are shown. *Lower:* PCR products from cortex and cerebellum DNA of indicated mouse strains. Top band represents the 550-bp product amplified from the recombined floxed *nrf1* allele. Bottom band represents the 250-bp product amplified from the nonrecombined allele. (*B*) Nrf1 antisense probe was used to detect Nrf1 expression on coronal sections of nrf1-/lox (control) and CamK2Cre; nrf1-/lox (Nrf1BKO) brains at 4 mo. Abundance of Nrf1 transcripts is shown using pseudocolorized computer images. (*C*) Immunofluorescence labeling of Nrf1 protein in hippocampus of control and Nrf1BKO brains.

Fig. S3. Apoptosis and accumulation of ubiquitinated proteins in Nrf1BKO mice and Nrf1-deficient neurons. (*A*) Fluoro Jade B staining in control and Nrf1BKO brains. (*B*) Western blot analysis of activated caspase-9 expression in control and Nrf1BKO brains. *Lower:* Actin levels in each lane. (*C*) Fluorescent immuno-histochemical double-staining of NeuN (green) and cleaved caspase-3 (red) in control and Nrf1BKO brains. Note colocalization of cleaved-caspase-3 and NeuN signal in the merged image of Nrf1BKO brain. (*D*) GFAP staining of brains at 6 mo. (*E*) Immunofluorescence of ubiquitin and NeuN in control and Nrf1BKO brain sections stained with anti-ubiquitin (red) and with anti-NeuN (green). Colocalization of ubiquitin and NeuN signal is shown in the merged image. (*F*) Representative immunohistochemical staining for ubiquitin in Nrf1$^{flox/flox}$;Cre-ERT2 neuronal cultures after 5-d treatment with DMSO or tamoxifen (4HT). (*G*) Representative TUNEL staining in Nrf1$^{flox/flox}$;Cre-ERT2 neuronal cultures after 9-d treatment with DMSO or tamoxifen (4HT).
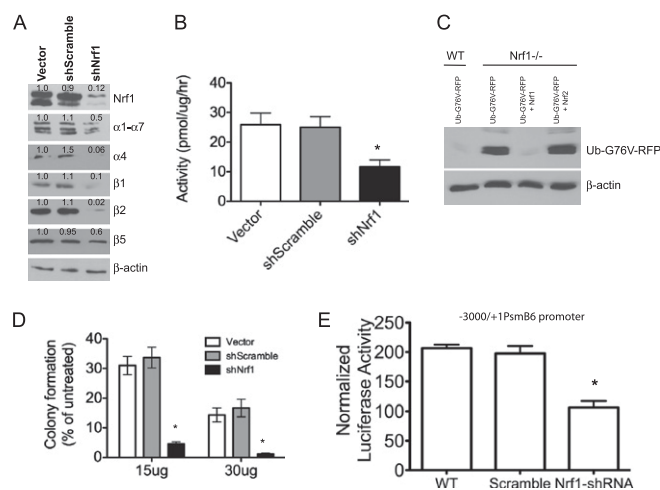
Fig. S4. Nrf1 regulates proteasome expression and activity. (*A*) Western blot analysis of Nrf1 and various proteasomal protein subunits in parental 293 cells transduced with vector, shScramble, and shNrf1 virus. Densitometric quantitations of band intensities are shown. (*B*) Comparison of chymotrypsin-like activity in lysates from cells expressing knockdown shRNA, shScramble, and vector only. Mean values ± SEM are from triplicate cultures of control and knockdown cells. *$P < 0.05$. (*C*) Immunoblot analysis of Ub^{G76V}-RFP in Nrf1$^{-/-}$ cells transfected with the Ub^{G76V}-RFP expression construct. Protein loading was evaluated by immunoblotting against β-actin. (*D*) Clonogenic survival assay of shScramble- and shNrf1-transduced cells treated with MG132 or DMSO vehicle. Data are normalized to the shScramble-transduced cells; means ± SEM, $n = 3$. *$P < 0.05$. (*E*) Activity of the PsmB6 luciferase reporter in parental 293 (Mock), shScramble-transduced, and cells stably expressing Nrf1 shRNA. All activities represent the mean of at least three independent experiments ± SEM. *$P < 0.05$.

Fig. S5. Oxidative stress is not induced by loss of Nrf1 in neurons. (*A*) Reduced/oxidized glutathione (GSH/GSSG), (*B*) GSH, and (*C*) GSSG levels of control, Nrf1BKO, and Nrf2 KO brains. Values are mean (*n* = 4 per group) ± SEM. *$P < 0.05$. (*D*) DCF fluorescence levels in Nrf1$^{+/+}$;Cre-ERT2 and Nrf1$^{flox/flox}$;Cre-ERT2 neuronal cells treated with tamoxifen (4-HT). Results are means ± SEM, *n* = 3 for each group. (*E*) Chymotrypsin-like activities in brain homogenates of wild-type and Nrf2 KO brains. Mean values ± SEM (control *n* = 6, Nrf2KO *n* = 4). $P \leq 0.05$.

Fig. S6. Network of differentially expressed genes in the proteasome pathway in Nrf1BKO brains. IPA was performed on microarray data comparing mRNA isolated from control and Nrf1BKO brains. Using 1,431 differentially expressed genes as the focus gene set, the proteasome pathway was identified as the highest-scoring pathway. The network is shown graphically. Genes within the network are represented as nodes. Red nodes denote up-regulation, and green nodes denote down-regulation in Nrf1 knockout brains. Arrows and lines connect interactions between genes within the network.

Table S1.   Primer list

Gene	Forward	Reverse
mPsmA6	AACGGAAAGCATTGGCTGTGT	TGCACAGCATGTCCACAGGAA
mPsmA7	CGGCCCTAATTGTGGGTTTTG	GTTTTTGCCACCTGACTGGAC
mPsmB6	GCAGGCTGGACCCTCAAGAA	CAAGCGGATCACCCCTCCACT
mPsmB7	TCTGGCTCCTTGGCAGCAATG	GGCCAAGCCTGGTCCCTTTCT
mPsmB2	CCTGGCTGGCTATGACGAGCA	CGGACACTGAAGGTGGGCAGA
mPsmB5	AACCACCACCCTGGCCTTCAA	AGCCAACAACCGCTCCCAGAA
mPsmB1	TTGACCCAGTGGGCTCTTACC	CTTTCACCAGCCTCATGGCTC
mPsmC2	GATGTTGGTGGCTGTAAGGAA	CCCAATAACTCGAATGAAGCA
mPsmC3	CCACTCACGGAAGATGAATGT	TCAACTTTAGCCCTCAGACCA
mPsmC5	CGGCCTGGACAAGCAGATCAA	CTGGGGGTCCGTAGAGCAGGA
mPsmD3	CGGCTCTGTGCCTGGTCTCTG	TCCCAGCTGCTGCCTCCTCTT
mPsmD11	AGGCAGACAGAAGCATTGAAA	GGTCCAAAATCCCATGAAACT
mPsmD4	TCTCCTATTCTGGCTGGTGAA	CATGCTGCTTAGGTCTGGAAG
mPsmD12	GTGGCCGAACCTGGGGTACAG	TCTTGAAGCCGCCCTTCCTTG
ChIP primers		
−3000 *PsmB6*	TCGTGAACGACAGAGTGCTT	CACCTGGTTGGTTGTGTTTCT
−2500 *PsmB6*	CCAGCAACAAAAGCAAAACA	CTCTAGCCCCGGCTATATGA
−2000 *PsmB6*	TTGCCCAAAGCTGAAACTT	AGCCAGGGCTACACAGAAAA
−1500 *PsmB6*	CCCTCTTCTGGTGTGTGCTGA	TGGGACTTTTGGGATAGCAC
−1000 *PsmB6*	GCTATCCCAAAAGTCCCACA	GGTCCCACAAGAACTTTTGAA
−500 *PsmB6*	CAACCTGTTAACATTGCGACA	ACTTCCCGGTTTTCCCAGTCTG
NQO1	GCAGTTTCTAAGAGCAGAACG	GTAGATTAGTCCTCACTCAGCCG
LDH	GCAAAGCCAGTACTCTTTCTG	CGGCCATGTTCTGAATCCAAG