Loss of nuclear factor E2-related factor 1 in the brain leads to dysregulation of proteasome gene expression and neurodegeneration

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The ubiquitin–proteasome pathway plays an important role in the pathogenesis of neurodegeneration, but mechanisms controlling expression of components in this pathway remain poorly understood. Nuclear factor E2-related factor 1 (Nrf1) transcription factor has been shown to regulate expression of antioxidant and cytoprotective genes. To determine the function of Nrf1 in the brain, mice with a late-stage deletion of Nrf1 in neuronal cells were generated. Loss of Nrf1 leads to impaired proteasome function and neurodegeneration. Gene expression profiling and RT-PCR analysis revealed a coordinate down-regulation of various proteasomal genes including PsmB6, which encodes a catalytic subunit of the proteasome. Transcriptional analysis and chromatin immunoprecipitation experiments demonstrated that PsmB6 is an Nrf1 target gene. These findings reveal Nrf1 as a key transcriptional regulator required for the expression of proteasomal genes in neurons and suggest that perturbations of Nrf1 function may contribute to the pathogenesis of neurodegenerative diseases.

Results

Generation of Nrf1 Brain-Specific Conditional Knockout. In situ hybridization (ISH) of Nrf1-specific probe was widespread in the mouse brain. High levels were detected in the cortex, cornu ammonis (CA) subregions and dentate gyrus of the hippocampus, and choroids plexus in adult mice brains (Fig. S1A). Nrf1 immunoreactivity was prominently detected in the mouse brain in a pattern similar to that seen by ISH, and double-labeling showed overlap between Nrf1 staining and cells positive for the neural marker NeuN (Fig. S1B). Cultured cortical neurons also showed strong immunostaining for Nrf1 (Fig. S1C). These results indicate that Nrf1 is highly expressed in neurons. To address the role of Nrf1 in the adult brain, we generated mice with deletion of Nrf1 selectively in the brain to bypass embryonic lethality in constitutive Nrf1 knockout mice. The Nrf1 flox mouse was crossed with the Calcium-calmodulin-dependent Protein Kinase Type 2–Cre (Camk2Cre) transgenic mouse to generate Camk2Cre;Nrf1−/−/floxed animals, herein referred to as Nrf1BKO. Cre expression in Camk2Cre mice has been shown previously to occur at 1 mo of age and to be confined primarily to differentiated neurons in the forebrain (15). In accord with this, the recombined Nrf1 allele was detected in the cortex but not the cerebellum of Nrf1BKO mouse (Fig. S2A). In situ mRNA hybridization using Nrf1-specific riboprobe showed Cre/loxP-mediated Nrf1 deletion in the cortex and hippocampus of Nrf1BKO brain (Fig. S2B). Disruption of Nrf1 was further verified by immunofluorescence staining in the hippocampus (Fig. S2C).


The authors declare no conflict of interest.

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Nrf1BKO Mice Show Age-Dependent Forebrain Atrophy. Nrf1BKO mice were born at the expected ratio without evidence of developmental defects, and they were indistinguishable from their control littermates at weaning. However, a number of behavioral abnormalities were observed in the course of maintaining these animals. At 3 to 4 mo of age, Nrf1BKO animals showed abnormal hindlimb-clasping reflexes that are often observed in mouse models of neurodegeneration (Fig. L4) (16). To examine whether Nrf1BKO mice show age-dependent neuronal loss, we compared brain weights of Nrf1BKO and control mice. No significant differences could be observed at weaning, but Nrf1BKO brain weights were dramatically lower by 6 mo (Fig. 1B). Gross examinations showed that reduction was attributable to forebrain atrophy (Fig. 1C). Histologic evaluation indicated that the thickness of the cortex and hippocampus in Nrf1BKO mice was reduced (Fig. 1D) as a result of decreased number of NeuN-positive cells in Nrf1BKO brain (Fig. 1E). Neuronal loss was quantitatively assessed by stereological analysis. Nrf1BKO mice showed a 25% and 38% (P < 0.05, respectively) reduction in the volume of the cortex at 3 and 6 mo, respectively (Fig. 1F). These results indicate that Nrf1BKO mice suffer significant neuronal loss.

Apoptotic Cell Death and Accumulation of Ubiquitin in Neurons of Nrf1BKO Mice. To identify degenerating neurons, Fluoro Jade B staining was done. Fluoro Jade B staining was observed in the cortex and hippocampus of Nrf1BKO brains compared with controls (Fig. S3A). Numerous apoptotic nuclei were seen in H&E-stained sections of Nrf1BKO brains compared with control brains (Fig. 2A). Consistent with this, Nrf1BKO brains showed activated caspase-3 staining (24 ± 7 positive cells per 20x field), whereas none was detected in controls (Fig. 2A, Inset). In addition, activation of caspase-3 was colocalized to neurons (Fig. S3C). Activated caspase-9, a protease involved in the upstream regulation of apoptosis, was also detected in Nrf1BKO brains (Fig. S3B). Increased GFAP immunostaining was also observed (Fig. S3D), suggesting that astrogliosis accompanies neuronal damage. Interestingly, numerous ubiquitin-positive cells were detected as early as 1 mo of age, and they were primarily in the cerebral cortex and CA subfields of the hippocampus (Fig. 2B). Double-staining experiments showed that ubiquitin-immunoreactive cells are also positive for NeuN, indicating that neurons were affected (Fig. S3E). Western blotting showed an increase in high-molecular-weight ubiquitin–protein conjugates in Nrf1BKO brains compared with controls (Fig. 2C).

To determine whether abnormalities observed were a direct effect of Nrf1 loss of function in neurons, we analyzed neuronal cells derived from Nrf1<sup>flox/flox</sup> mice bred to mice expressing tamoxifen-inducible Cre recombinase (Cre-ERT2). This strategy allows the inactivation of Nrf1 in cells by treatment with 4-hydroxytamoxifen (4HT). Quantitative RT-PCR analysis and immunoblotting to verify the efficiency of tamoxifen-induced recombination in cultures of Nrf1<sup>ox/ox</sup>/Cre-ERT2 neuronal cells showed that Nrf1 expression was markedly reduced after 72-h treatment (Fig. 3A). After 5 d of treatment, Nrf1<sup>flox/flox</sup>/Cre-ERT2 neuronal cultures showed fourfold increase in number of ubiquitin immunoreactive cells compared with Nrf1<sup>flox/flox</sup>/Cre-ERT2 cultures treated with vehicle (Fig. 3B). By 7 d, most of the cells in 4HT-treated Nrf1<sup>flox/flox</sup>/Cre-ERT2 cultures were ubiquitin positive (Fig. 3B and Fig. S3F). TUNEL labeling did not reveal a significant difference between vehicle-treated Nrf1<sup>flox/flox</sup>/Cre-ERT2 cells and Nrf1<sup>+/+</sup>/Cre-ERT2 at the different time points investigated. However, a significant increase in apoptosis was detected in Nrf1<sup>flox/flox</sup>/Cre-ERT2 cells compared with Nrf1<sup>+/+</sup>/Cre-ERT2 cells after 9 d of 4HT treatment (Fig. 3C and Fig. S3G). These results suggest that the defects in Nrf1BKO brains are directly associated with Nrf1 deficiency in neurons, and accumulation of ubiquitin is not a consequence of cell death.

Impaired Proteosomal Function in Nrf1BKO Brains. The accumulation of ubiquitinated proteins suggested that proteasome impairment could be involved in neuronal damage observed in the Nrf1BKO mice. Indeed, Nrf1BKO brains showed a 30% decrease in chymotrypsin-like activity compared with controls (Fig. 4A). Trypsin-like and caspase-like activities were also diminished by 20% and 50%, respectively in Nrf1BKO brains (Fig. 4A). To confirm these results, in-gel assay to measure proteasome activity was performed. The chymotrypsin-like activities of both the 20S and the 26S proteasomes were markedly reduced in Nrf1BKO brains compared with controls (Fig. 4B). Similarly, in-gel measurements of trypsin-like and caspase-like activities of Nrf1BKO brains were also diminished compared with controls (Fig. 4C). Immunoblotting against actin showed that equal amounts of brain lysates were used for the in-gel studies (Fig. 4D).
Nrf1 Deficiency in Cells Leads to Impaired Proteasome Function and Hypersensitivity to Proteasome Inhibition. We next examined whether proteasome function is also impaired in primary mouse embryonic fibroblasts (MEFs) from Nrf1−/− animals. A significant decrease in proteasome activity was observed in Nrf1−/− MEF cells in comparison with wild-type MEF cells (Fig. 3A). In line with these data, a lentiviral shRNA-mediated system that prevents the contribution of Nrf1 degrading in Nrf1−/− cells (Fig. 3B). To measure intracellular proteasome activity, we monitored steady-state levels of red fluorescent protein (RFP) in cells transfected with UbG76V-RFP expression plasmid. The UbG76V-RFP is a highly unstable protein targeted for degradation by the proteasome and is normally present at low levels in transfected cells unless proteasome activity is impaired (17). Although both wild-type and Nrf1−/− MEF cells transfected with UbG76V-RFP showed similar expression of RFP mRNA (Fig. 3B), RFP fluorescent levels were markedly elevated in Nrf1−/− MEF cells compared with wild-type cells (Fig. 3C). In contrast, fluorescent levels in wild-type cells were close to background levels (Fig. 3C). Confirming the instability of the UbG76V-RFP protein in wild-type cells, Nrf1−/− cells showed increased UbG76V-RFP protein levels (Fig. 3D). Transfection of wild-type Nrf1 cDNA, but not a bZIP deletion mutant of Nrf1, was able to restore UbG76V-RFP clearance in Nrf1−/− cells (Fig. 3C and D). In addition, transfection of Nrf2 also did not restore UbG76V-RFP clearance in Nrf1−/− cells, indicating that effects are specific to Nrf1 (Fig. S4C). To further assess the potential for a direct contribution of Nrf1 deficiency to proteasome impairment, clearance of UbG76V-RFP was also monitored in MEF cells rendered Nrf1 deficient. MEF cells generated from Nrf1lox/lox; Cre-ERT2 mice were treated with DMSO or 4HT for 72 h and then transfected with UbG76V-RFP. RFP fluorescent levels were significantly higher in 4HT-treated Nrf1lox/lox; Cre-ERT2 cells compared with the same cells treated with DMSO, as well as Nrf1−/−; Cre-ERT2 control cells treated with 4HT or DMSO (Fig. S5E).

To assess the functional effect of Nrf1 deficiency on proteolytic stress, we tested whether Nrf1−/− MEFs and Nrf1 knockout cells are sensitized to proteasome inhibition. Nrf1−/− MEF cells treated with epoxomicin showed a threefold increase in cell death compared with wild-type and Ndr2−/− cells (Fig. 5F). Similarly, treatment with MG132 caused increased cell death and reduced the colony-forming capability of Nrf1 knockdown cells compared with scramble cells (Fig. S4D).

Nrf1BKO Brains Do Not Show Increased Oxidative Stress. Because the degradative capacity of the proteasome may be compromised by oxidative stress and Nrf1 has been shown to be involved in the antioxidant pathway, we examined whether proteasomal defects in Nrf1BKO brains are associated with increased oxidative stress. The ratios of reduced to oxidized glutathione (GSH/GSSG) levels in control and Nrf1BKO brain tissues were similar (Fig. S5A), and no significant differences in GSH and GSSG levels were seen in control and Nrf1BKO brain tissues (Fig. S5A and C). As a positive control, we examined GSH and GSSG levels in Nrf2 knockouts. GSH/GSSG ratio was twofold lower in Nrf2 knockout brains (Fig. S5A). Consistent with the primary role of Nrf2 in coordinating cellular defense against oxidative stress in neurons (18), the reduction in GSH/GSSG ratio in Nrf2 knockout brains was associated with an elevation in GSSG level (Fig. S5C). We next measured intracellular reactive oxygen species (ROS) levels in neuronal cells. No increase in ROS levels was detected in Nrf1lox/lox; Cre-ERT2 neurons treated with 4HT compared with neurons treated with vehicle (Fig. S5D). Although Nrf2 knockout brains showed evidence of oxidative stress, impairment in proteasome activity was not detected (Fig. S5E). Together, these

Fig. 3. Accumulation of ubiquitinated proteins and apoptosis are induced by loss of Nrf1 in neurons. (A) Nrf1 expression in Nrf1lox/lox; Cre-ERT2 neuronal cultures treated with DMSO or 4HT. Nrf1 mRNA levels were measured by quantitative RT-PCR. Data were normalized to the 18s RNA; means ± SEM, n = 3. *P < 0.05. Inset: Western blot analysis of Nrf1 in DMSO and 4HT treated Nrf1lox/lox; Cre-ERT2 neuronal cells. (B) Quantitation of ubiquitin immunostaining in Nrf1−/−; Cre-ERT2 neuronal cultures at various days after treatment with DMSO or 4HT. (C) Quantitation of TUNEL labeling in Nrf1−/−; Cre-ERT2 neuronal cultures at various days after treatment with DMSO or 4HT. (D) Amounts of protein used in in-gel assays were evaluated by β-actin immunoblotting.

Fig. 4. Proteasome activity is impaired in Nrf1BKO brains. (A) Proteolytic activities of the proteasome subtypes from 1-mo-old brains. Mean values ± SEM (control n = 6, Nrf1BKO n = 6). *P < 0.05. (B) Measurement of chymotrypsin-like activity by in-gel assay with fluorogenic Suc-LLVY-AMC as a substrate. Fluorescence from free AMC was visualized on a UV transilluminator. Six control and six Nrf1BKO samples were analyzed, and two representative samples from each genotype are shown. Upper: The 26S proteasome that appears in two forms—RP1CP, 20S CP with one bound regulatory particle; and RP2CP, 20S CP with two bound regulatory particles. Lower: The faster-migrating free 20S core particle. Densitometric quantitations for 26S and 20S levels are shown. (C) In-gel analysis of trypsin-like and caspase-like activities in control and Nrf1BKO brains. Two representative samples from each genotype are shown. Densitometric quantitations are shown. (D) Amounts of protein used for in-gel assays were evaluated by β-actin immunoblotting.
Brains and Cells.

Quantitative RT-PCR. Values are expressed as threshold cycle (Ct) corrected for 18s rRNA levels. Mean values of viability between wild-type, Nrf1−/− activities in MEF cells. Mean values analysis of RFP

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results suggest that proteasome dysfunction is not linked to oxidative stress in Nrf1BKO brains.

Proteasome Gene Expression Is Down-regulated in Nrf1-Deficient Brains and Cells. To better understand the Nrf1BKO phenotype, microarray transcriptional profiling was done. Frontal cortices of Nrf1BKO and matched controls were analyzed by oligonucleotide arrays. A total of 1,149 genes were identified by ANOVA (P < 0.05) as differentially expressed. Among these genes, 574 were underexpressed, and 575 genes were overexpressed in Nrf1BKO frontal cortex compared with control. This dataset was then analyzed using Ingenuity Pathway Analysis (IPA) software to identify processes that might be affected in the Nrf1 brain knockouts. The top canonical pathway identified was associated with proteasome function (Fig. S6). Other top-scoring networks identified also included High-affinity IgE receptor (Fc-epsilon RI) signaling, axon guidance, and inflammatory response. The increase in inflammatory response is consistent with the ongoing neurodegeneration and astrocytic response observed in the Nrf1BKO brains. However, the relevance of Fc-epsilon RI signaling and axon guidance is not clear and was not pursued further here. On the basis of proteasomal dysfunction exhibited by Nrf1BKO brains, genes representative of the 20S core and 19S regulatory complex of the proteasome were further studied. Quantitative RT-PCR showed down-regulated expression of genes encoding various subunits of the 20S core, as well as the 19S regulatory complex in Nrf1BKO brains (Fig. 6A). In accord with RT-PCR results, Western blotting showed decreased levels of α-subunits and catalytic β-subunits of the 20S core, as well as RPT2 and RPT5 of the 19S complex (Fig. 6B). To determine whether expression of some of the above proteasome genes was directly linked to Nrf1, we analyzed their expression in Nrf1−/− Cre-ERT2 and Nrf1+/− Cre-ERT2 neurons after tamoxifen treatment. Consistent with results obtained from knockout brains, expression of PsmA6, -B2, -B6, B7, and -D11 was decreased in Nrf1−/− Cre-ERT2 neurons compared with tamoxifen-treated Nrf1+/− Cre-ERT2 neurons (Fig. 6C). In addition, the knockdown of Nrf1 in 293 cells resulted in a reduction of α-subunits and catalytic β-subunits compared with cells transduced with vector or with a scrambled shRNA (Fig. S4A). Together these data suggest that proteasome dysfunction in Nrf1BKO can be explained by alterations in proteasome subunit content.

PsmB6 Is a Direct Nrf1 Target Gene. We next determined whether proteasome genes are directly under the control of Nrf1. On the basis of our expression results above, PsmB6 (encoding the catalytic β-subunit) was chosen as a representative target gene. The promoter region of PsmB6 gene (3.0 kb) was isolated from genomic DNA by PCR amplification and cloned into the pGL3Basic luciferase reporter plasmid. Nrf1 activated the PsmB6 luciferase reporter in a dose-dependent manner (Fig. 7A). PsmB6 luciferase reporter expression is Nrf1 dependent, we compared expression of the PsmB6 luciferase reporter plasmid in wild-type and Nrf1 knockout MEFs. Luciferase expression in Nrf1 knockout MEFs was threefold lower compared with wild-type and Nrf2 knockout MEFs (Fig. 7B), and low expression of the reporter
was rescued by cotransfection of Nrf1 expression plasmid (Fig. 7B). Cotransfection of bZIP deletion-mutant Nrf1, or Nrf2, did not rescue promoter activity (Fig. 7B). To further substantiate the role of Nrf1 in PsmB6 activation, luciferase reporter activity was reduced by 50% in Nrf1 knockdown cells (Fig. 7C). Sequence inspection revealed a perfect consensus Nrf1 binding site at -38 nt. Next, a deletion of the ARE in the -38 region was generated to verify its role in the regulation of the PsmB6 gene promoter. Both basal and activated luciferase expression by Nrf1 cotransfection was blunted when the ARE region was deleted (Fig. 7D). These data provide support that Nrf1 directly regulates PsmB6 expression in vivo.

**Discussion**

Although Nrf1 has been shown to regulate antioxidant gene expression, additional roles of Nrf1 in development and cellular function were investigated. Nrf1 is highly expressed in neural tissues, but little is known about its function in the brain. Here we show that CaMK2cre-directed conditional knockout of Nrf1 (Nrf1BKO) leads to neuronal apoptosis and age-dependent brain atrophy. Our data also indicate that Nrf1 deficiency in neural cells does not induce measurable oxidative stress in brain tissues of Nrf1BKO mice. Thus, the neurodegenerative phenotype in Nrf1BKO mice is not mechanistically linked to oxidative stress. Instead, the knockout of Nrf1 in both mouse brains and cells produces defects in proteasome function and transcriptional regulators that are involved (22). Transcriptional regulation of many cytoprotective genes is regulated.

**Fig. 6.** Nrf1 deficiency in mouse brains leads to coordinate down-regulation of proteasome genes. (A) Comparison of proteasome gene expression by quantitative RT-PCR analysis. Mean values ± SEM (n = 3 for each genotype). **P** < 0.05. (B) Levels of proteasome subunits analyzed by Western blotting. Densitometric quantitations of band intensities are shown. (C) Quantitative RT-PCR analysis of mRNA encoding proteasomal genes in Nrf1+/−Cre-ERT2 and Nrf1fox/fox/Cre-ERT2 neuronal cultures after tamoxifen treatment. Results are means ± SEM, n = 4 for each group. **P** < 0.05.

**Fig. 7.** Nrf1 regulates PsmB6 gene. (A) Transactivation of the mouse PsmB6 promoter by Nrf1. PsmB6 luciferase reporter was transfected along with vector, Nrf1, or Nrf2 cDNA into 293 cells. Activities represent the mean of at least three independent experiments ± SEM. **P** < 0.05. (B) Activity of the PsmB6 luciferase reporter in wild-type, Nrf1−/−, Nrf2−/−, or Nrf1−/− fibroblasts expressing Nrf1 cDNA, Nrf2 cDNA, or Nrf1 cDNA containing a deletion in the bZIP domain. Results represent the mean of at least three independent experiments ± SEM. **P** < 0.05. (C) Chromatin immunoprecipitation of PsmB6 promoter in vivo. Graph represents real-time PCR amplification of chromatin template precipitated with either anti-Nrf1 or rabbit preimmune IgG. Primers were targeted to various regions spaced 500 bp apart along the PsmB6 promoter. NQO1 and LDH promoters were used as positive and negative controls, respectively. Nonimmunoprecipitated chromatin (1%) was used as an input control. The open box indicates the location of a consensus binding site for Nrf1 in the PsmB6 promoter. (D) Activity of wild-type PsmB6 promoter and mutant promoter containing a deletion of the −38 ARE site. Reporter constructs were transfected along with vector or Nrf1 cDNA into MEF cells. Activities represent the mean of three independent experiments ± SEM. **P** < 0.05.

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through the ARE (23). For example, AREs control expression of both the catalytic and regulatory subunits of glutamate cysteine ligase that are involved in the biosynthesis of GSH (24, 25). The ARE also regulates phase I and phase II detoxification enzymes and multidrug resistance-associated transporters. In addition to oxidative stress-related genes, AREs have also been identified in promoters of a number of proteasomal genes (26, 27). Although Nrf2 has been shown to activate expression of proteasomal genes, proteasome activity was affected in Nrf2 knockout brains. This suggests that proteasome genes expression is not dependent on Nrf2 in neural cells. One possible explanation is that the level of Nrf2 expression may not be high enough in cortical neurons. Alternatively, the repertoire of target genes regulated by Nrf1 is uniquely different from Nrf2. This possibility would also explain the absence of oxidative stress in Nrf1BKO brains. Our data indicate that Nrf1 is a main regulator of proteasomal gene expression. On the basis of our gene expression profiling data, it seems that the proteasome pathway is one of the key processes affected by loss of Nrf1 in neurons. Interestingly, however, ARE-driven genes involved in oxidative stress did not appear in our microarray analysis. In line with a role for Nrf1 in proteasome gene expression, mRNA analysis and Western blotting showed a coordinate down-regulation of both α- and β-catalytic subunits of the 20S subunits, as well as components of the 19S regulatory subcomplex in Nrf1BKO brains. The marked accumulation of ubiquitinated protein aggregates suggests that the expression of proteasome genes is sufficiently diminished by loss of Nrf1 to affect constitutive proteasome function in Nrf1BKO brains. In further support of the requirement for Nrf1 in proteasomal gene expression, proteasome expression was down-regulated by tamoxifen-induced knockout of Nrf1 in neurons, and Knockdown of Nrf1 activity by shRNA in 293T cells impaired proteasome expression and function in a parallel fashion. Our data also demonstrate that PsmB6, chosen as a representative target gene on the basis of our gene expression analysis, is directly regulated by Nrf1. Sequence inspection also revealed putative AREs in the 5’ flanking regions of various proteasomal genes, such as PsmA5, PsmB2, PsmB1, and PsmC3. However, further studies are required to examine whether Nrf1 also contributes directly to expression of these genes. Aside from its role in constitutive expression of proteasome genes, we have also demonstrated that Nrf1 is required for inhibitor-induced proteasome gene expression (28). On the basis of these findings, we propose that Nrf1 is essential for expression of mammalian 26S proteasome under basal as well as stress-induced conditions.

In summary, this study evaluated the role of Nrf1 in vivo in the adult brain. The results of our present study clearly demonstrate that Nrf1 is required for normal expression of proteasome genes in neural cells, and loss of Nrf1 in neurons causes neurodegeneration. These findings provide information on the role of Nrf1 beyond the oxidative stress response. Because neuronal degeneration is frequently observed in patients and animal models with defective proteasome function, these findings raise the possibility that Nrf1 signaling might play a more general role in neurodegenerative diseases. The Nrf1BKO mouse may provide a useful model to examine the involvement of proteasome dysfunction in the pathogenesis of neurodegenerative diseases.

Materials and Methods

See SI Materials and Methods for greater detail.

Transcriptional Profiling and Quantitative RT-PCR. Profiling was done on Affymetrix oligonucleotide arrays. Primer sequences for PCR are listed in Table S1. Quantitative RT-PCR data was calculated as $2^{-\Delta\Delta Ct}$ (29).

Transient Transfection and Luciferase Assays. Cells were transfected using Lipofectamine and extracts were measured with Luciferase Reporter Assay Kit (Promega).

Primary Neuron Cultures. Cultures were generated from cortices of embryonic day 14–15 embryos.

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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 PSMel-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. Protease substrates Suc-Leu-Leu-Val-Trp-7-amino-4-methylcoumarin, Boc-Leu-Arg-Arg-7-amino-4-methylcoumarin, and Asp-7-amino-4-methylcoumarin were from Enzo Life Sciences. Biotinylated anti-rabbit IgG and 3,3′-diaminobenzadine (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-floating 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 x 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 MgCl2, 4 mM ATP, and 1 mM DTU] using a polytron and centrifuged at 12,000 × 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-Trp-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-nLPlD-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- to 3-old control and Nrf1BKO mice were separated by native gel electrophoresis and assayed for 26S and 20S proteasome complexes by overlaying 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-nLPlD-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, brains 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 triluoromethanesulfonate to measure oxidized glutathione (GSSG) level. Protein samples were cleared by centrifugation at 13,000 × 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)-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 cell 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 software.
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 at 37 °C 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% 1-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, 0.5 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 RNA levels as endogenous controls. Relative expression was calculated as 2^(-ΔΔCt test gene – ΔΔCt 18s).

**Western Analysis.** Protein lysates were made using Nonident 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 × 10^3) 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 GGGCGCCACCTTCTCTGCTA and TGAATGGCTCATCGCCATCCCAT, respectively, and cloned into the Nhel and Xhol 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 BamHI sites of 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% CO2 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% formaldehyde 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’s t test or one-way ANOVA were used. \( P \) values of <0.05 were considered statistically significant.


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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 NeuN (red) in the cortex. Merged image shows costaining of Nrf1 and NeuN. (C) Immunofluorescent staining of Nrf1 (green) and NeuN (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 loxP 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 immunohistochemical 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<sup>lox/lox</sup>;Cre-ERT2 neuronal cultures after 5-d treatment with DMSO or tamoxifen (4HT). (G) Representative TUNEL staining in Nrf1<sup>lox/lox</sup>;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<sup>G76V</sup>-RFP in Nrf1<sup>−/−</sup> cells transfected with the Ub<sup>G76V</sup>-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, Nrf1KO, 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+/+;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 ≤ 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

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**ChIP primers**

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