Supporting Information

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SI Materials and Methods

Genotyping. Genotyping of mice was performed by PCR assay on DNA isolated from mouse tail using a commercial kit (DNeasy Blood & Tissue Kit; Qiagen). PCR amplification was performed with 1 μg of purified DNA on a PTC200 Gradient Cycler (MJ Research) using the following PCR settings: 33 cycles, 3 min of denaturation at 94 °C, 60 s of annealing at 60 °C, and 60 s of extension at 72 °C. The following primer pairs were used: GS-forward (for): 5′-GCT TAG GAT GGG TTA CTC TTC CAA GG-3′ and GS-reverse (rev): 5′-ATC ATC ATC TCC CTT CTC CCA TTC C-3′, and albumin promoter-controlled Cre recombinase gene (Alb-Cre) for: 5′-CTG TCA CTT GGT CGT GGC AGC-3′ and Alb-Cre rev: GTC CAA TTT ACT GAC CGT ACA-3′. PCR products were visualized on a 1.5% (wt/vol) ethidium bromide-containing agarose gel using a gel documenter (Vilbert Lourmat).

O-Maze. A 5.5-cm-wide annular runway was constructed using gray PVC. It had an outer diameter of 46 cm and was placed 40 cm above the floor. The two opposing 90° closed sectors were protected by 11-cm-high inner and outer walls of gray PVC, whereas the remaining two open sectors had a border of 5 mm. Animals were released in one of the closed sectors and observed for 10 min. Mice were allowed to adapt to the new environment before they advanced onto an unprotected runway. Time spent in protected and unprotected zones and number of transitions between the areas were assessed.

Tissue Sampling. Brain tissue was prepared from the cerebellum, hippocampus, and cerebral cortex. The cerebral cortex was further dissected into somatosensory and piriform cortical areas, which can be separated accurately due to visible morphological characteristics. For this procedure, the forebrain hemisphere was placed in ice-cold PBS (pH 7.2, spiked with frozen pellets of saline) in a Petri dish under a dissecting microscope (Olympus SZX 10). While holding the hemisphere at the remaining brainstem, the forebrain was cut in several frontal slices, and the desired brain regions were microdissected from these slices according to Hof et al. (1). The dissected cortical regions were sampled in precooled Eppendorf cups and frozen in liquid nitrogen until further processing.

For measuring GS expression in muscle tissue, we dissected the ischiocrural and anterior thigh muscle bellies.

Slice Preparation for Immunofluorescence Analysis. For immunofluorescence analysis of liver and brain tissue, animals were killed by i.p. injection of pentobarbital (70 mg/kg) and perfused with 20 mL of physiological saline, followed by perfusion with 250 mL of buffered paraformaldehyde [4% (wt/vol), pH 7.2, 4–6 °C] or Zamboni's fixative [4% (wt/vol) paraformaldehyde and 15% (vol/vol) saturated picric acid in 0.1 M PBS, pH 7.2, 4–6 °C]. Tissue was immediately dissected, submerged in 20% (wt/vol) sucrose in PBS (24 h at 4 °C) until complete saturation, and finally frozen in precooled 2-methylbutane (Sigma–Aldrich) at −40 °C before being sliced into 50-μm-thick sections on a cryotome (Frigomobil; Leica).

Immunofluorescence Analysis. For immunofluorescence of mouse brain or liver, the following primary antibodies were used for immunostaining: 8-OH(d)G (mouse, monoclonal; QED Bioscience), GFAP (rabbit, polyclonal; Sigma–Aldrich), GS (mouse, monoclonal; BD Biosciences), Iba1 (rabbit, polyclonal; WAKO Chemicals GmbH), rhesus family B glycoprotein (goat, polyclonal; Abcam), ornithine aminotransferase (rabbit, polyclonal; Abcam), or calbindin 28k (Sigma–Aldrich). All antibodies, except anti-calbindin (1:10,000), were diluted 1:500 in PBS containing 0.1% saponin (Sigma–Aldrich) and 2% goat serum (Vector Laboratories) or 5% (wt/vol) BSA. Primary antibodies were labeled with fluorochrome-coupled anti-mouse or anti-goat Cy3 and anti-rabbit FITC antibodies (1:500). Slices were incubated in antibody solution for 2 d at 4 °C under gentle agitation, followed by repeated washing with PBS, and were finally mounted on microscope slides using Fluoromount-G (Southern Biotech). Confocal laser scanning microscopy was performed using an LSM510meta microscope (Zeiss). Wide-field fluorescence microscopy was performed using a Cell Observer Z1 microscope (Zeiss).

Histochemistry. For H&E staining, cryofixed liver slices (7 μm) were treated twice with xylol, each for 10 min, followed by three consecutive incubations in ethanol [100% (vol/vol), 90% (vol/vol), and 70% (vol/vol), respectively]. Slices were stained using Haematoxylin Solution Gill Nr. 3 (Sigma–Aldrich) for 1.5 min. After staining, slices were briefly washed in HCl (0.1% for 20 s) followed by water (10 min). Slices were then stained with Eosin Y solution (Sigma–Aldrich) for 3 min and briefly washed with water. Finally, slices were incubated consecutively in ethanol [70% (vol/vol), 90% (vol/vol), and 100% (vol/vol); each for 3 min] and xylol (5 min) and mounted using Fluoromount-G mounting media.

Western Blot Analysis. For protein analysis, brain or liver samples were homogenized with a pestle-homogenizer using 10 mmol/L Tris·HCl buffer (pH 7.4) containing 1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L $Na_4P_2O_7$, 1 mmol/L EDTA, 20 mmol/L NaF, 1 mmol/L sodium vanadate, 20 mmol/L β-glycerophosphate, and protease inhibitor mixture (Boehringer Mannheim). The lysates were centrifuged at 20,000 × *g* at 4 °C. Protein concentration of the supernatant was determined by Bradford assay (BioRad). For SDS gel electrophoresis and Western blot analysis, the supernatant was added to an identical volume of 2× gel-loading buffer containing 200 mmol/L DTT (pH 6.8). After heating at 95 °C for 5 min, the protein samples were subjected to gel electrophoresis (150–170 mg of protein per lane, 10% gels). Following electrophoresis, gels were equilibrated with transfer buffer [39 mmol/L glycine, 48 mmol/L Tris·HCl, 0.03% SDS, 20% (vol/vol) methanol]. Proteins were transferred to nitrocellulose membranes using a semidry transfer apparatus (Pharmacia). After the membrane was blocked with 5% (wt/vol) BSA in TBST [20 mmol/L Tris·HCl (pH 7.5) containing 150 mmol/L NaCl and 0.1% Tween 20], the membrane was incubated with the indicated primary antibody (3′-nitrotyrosine monoclonal and GS monoclonal, 1:5,000; GAPDH, 1:10,000) followed by a secondary HRP-coupled antibody (1:10,000). Finally, blots were washed with Tris-buffered saline and developed using Western-Lightning Chemiluminescence Reagent Plus (PerkinElmer). Densitometric analysis was performed with the Kodak Image Station 4400, using Kodak Molecular Imaging software.

Northwestern Blot Analysis. Blood-free perfused and frozen brain tissue was homogenized with a pestle-homogenizer using lysis buffer from an RNeasy Mini Kit (Qiagen). Total RNA was purified from mice brain areas according to the manufacturer's protocol. RNA concentration was estimated using a NanoDrop spectrophotometer. Northwestern blot analysis was performed using formaldehyde [2% (vol/vol)] containing agarose (0.8%) gels loaded with 1–2 mg of isolated total RNA. After electrophoresis, the RNA

was transferred overnight to a nitrocellulose membrane (Schleicher & Schuell) by capillary transfer. The gel was then soaked for 15 min in freshly prepared 50 mmol/L NaOH, followed by washing for 5 min in diethylpyrocarbonate-treated water before incubating the gel for 45 min in 20× SSC transfer buffer [3 M sodium acetate, 0.3 M sodium citrate (pH 7.0)]. At the end of the transfer, the membrane was baked for 2 h at 70 °C. To control the loading of RNA transfer, the membrane was stained with methylene blue solution [0.2% methylene blue in 0.3 mmol/L NaAc (pH 5.5)]. For detection of 8-OH(d)G immunoreactivity, the membrane was blocked in 5% (wt/vol) BSA solubilized in 20 mmol/L Tris·HCl (pH 7.5) containing 150 mmol/L NaCl and 0.1% Tween 20, and then incubated for 2 h with the anti–8-OH(d)G antibody (1:5,000) at room temperature after washing and incubation with HRP-coupled anti-mouse IgG antibody diluted 1:10,000 at room temperature for 2 h. Blots were developed and analyzed by densitometry on the Kodak Image Station 4400 as described above.

Real-Time PCR. Total RNA was isolated using an RNA extraction kit (Qiagen). The cDNA was generated using a QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR analysis was accomplished in 40 cycles on an AB7500 Sequence Detection System (Applied Biosystems) using SYBR Green as a reporter dye. Real-time PCR was performed using the following primer sequences (MWG Biotech): argininosuccinate lyase (ASL) rev: 5′-CCA GTG GCT ACT TGG AGG ACA G-3′ and ASL for: 5′-CC TCA AGG GAC TTC CAA GCA C-3′, carbamoyl phosphate synthetase 1 (CPS-1) rev: 5′-GAT ACT GGA GAC AGC ACA CCA ATC-3′ and CPS-1 for: 5′-TAT GTT ACC TAC AAT GGC CAG GAG-3′, orthinine transcarbamylase (OTC) rev: 5′-TAA GGA TTT CCC TTG CAA TAA AGG-3′ and OTC for: 5′-CCA GAG TCA AGT ACA GCT GAA AGG-3′, succinate dehydrogenase complex subunit A (SDHA) rev: 5′-GTG GGA ATC CCA CCC ATG T-3′ and SDHA for: 5′-CTT CGC TGG TGT GGA TGT CA-3′, Iba1 for: 5′-GTC CTT GAA GCG AAT GCT GG-3′ and Iba1 rev: 5′-CAT TCT CAA GAT GGC AGA TC-3′, and CD14 for: 5′-GGC GCT CCG AGT TGT GAC T-3′ and CD14 rev: 5′-TAC CTG CTT CAG CCC AGT GA-3′. Data were produced in duplicate for each gene. Target gene mRNA expression levels were normalized to mRNA expression levels of SDHA, which served as a housekeeping gene.

GS Activity Assay. For measuring GS activity, aliquots of 100 μL of tissue homogenate were incubated with 900 μL of reaction mixture at 37 °C. The reaction mixture contained 60 mmol/L L-Gln, 15 mmol/L hydroxylamine-HCl, 20 mmol/L Na-arsenite, 0.4 mmol/L adenosine diphosphate, 3 mmol/L $MnCl_2$, and 60 mmol/L imidazol-HCl buffer (pH 6.8) in a final volume of 1 mL. The reaction was initiated at 37 °C and terminated by the addition of 1 mL of stop-solution containing 0.2 mol/L trichloroacetic acid, 0.67 mol/L HCl, and 0.37 mol/L $FeCl_3$. The solution was cleared of protein by centrifugation at 20,000 × *g* at 4 °C, and the formed glutamyl hydroxamate was measured photometrically in the supernatant at 500 nm.

1. Hof PR, Young WG, Bloom FL, Belichenko PV, Celio M (2000) *Comparative Cytoarchitectonic Atlas of the C57BL/6 and 129/Sv Mouse Brains* (Elsevier, Amsterdam).

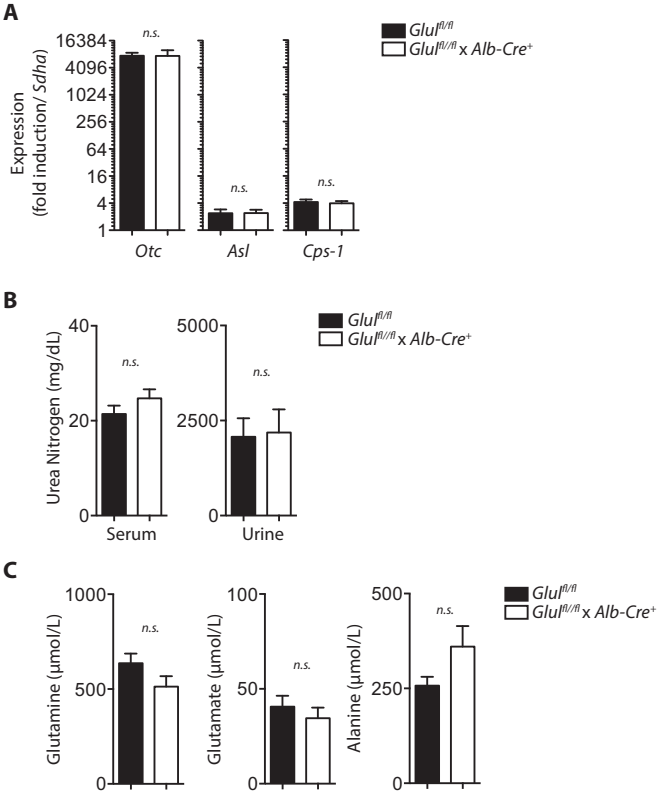

Fig. S1. GS is dispensable for the functional urea cycle. (*A*) Real-time PCR analyses of liver tissue harvested from $Glul^{fl/fl}$ and $Glul^{fl/fl}$ × *Alb-Cre*$^+$ mice of OTC (*Left*, *n* = 8–9), ASL (*Middle*, *n* = 8–12), and CPS-1 (*Right*, *n* = 8–9) were performed. (*B*) Urea nitrogen concentration was determined in $Glul^{fl/fl}$ and $Glul^{fl/fl}$ × *Alb-Cre*$^+$ mice: serum samples (*Left*, *n* = 12–14) and urine samples (*Right*, *n* = 7–9) from $Glul^{fl/fl}$ and $Glul^{fl/fl}$ × *Alb-Cre*$^+$ mice, respectively. (*C*) Serum Gln concentrations (*Left*), Glu concentrations (*Middle*), and Ala concentrations (*Right*) were assessed in $Glul^{fl/fl}$ and $Glul^{fl/fl}$ × *Alb-Cre*$^+$ mice (*n* = 11–13), respectively. n.s., not statistically significantly different.

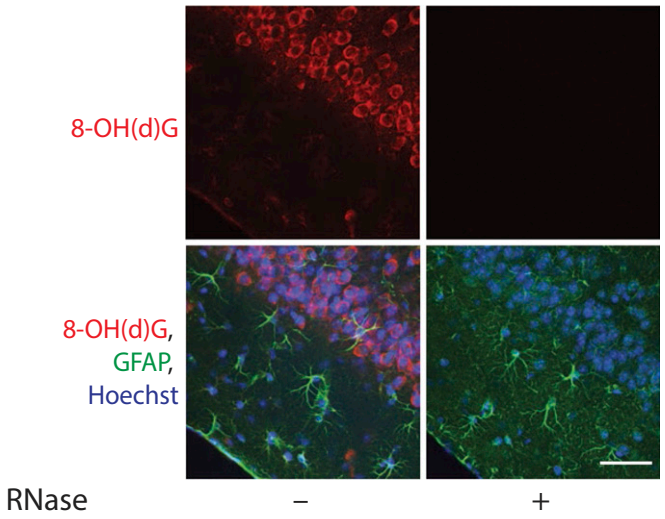

Fig. S2. Specificity control for the 8-OH(d)G antibody. A survey of 8-OH(d)G immunoreactivity in brain tissue from *Glul*$^{fl/fl}$ × *Alb-Cre*$^{+}$ mice is presented in the absence (*Left*) or presence (*Right*) of RNase (one representative of $n = 3$ is shown). (Scale bar: 50 μm.)

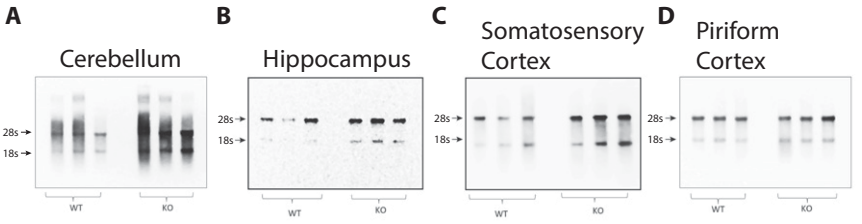

Fig. S3. Hepatic GS KO triggers RNA oxidation in brain tissue. Samples harvested from different brain regions of *Glul*$^{fl/fl}$ and *Glul*$^{fl/fl}$ × *Alb-Cre*$^{+}$ mice were tested for RNA oxidation expression using Northwestern blotting. Representative blots are presented for the cerebellum (*A*), hippocampus (*B*), somatosensory cortex (*C*), and piriform cortex (*D*).

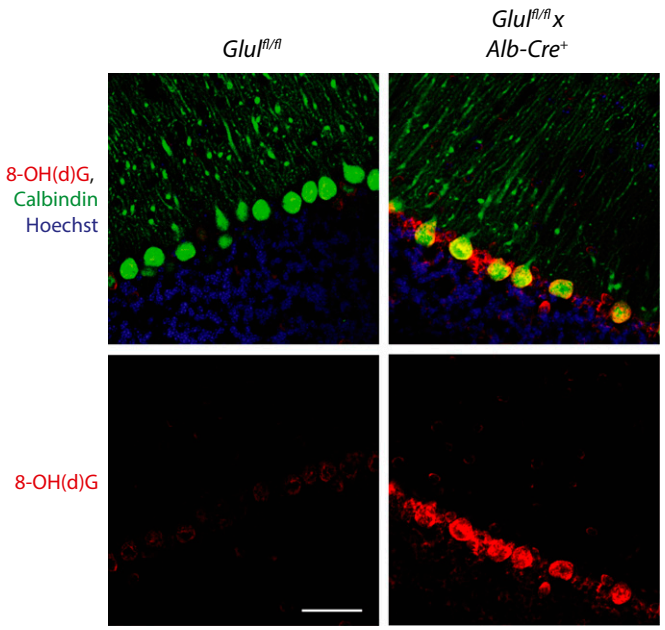

Fig. S4. Liver-specific deletion of GS induces RNA oxidation in cerebellar Purkinje cells. Survey of 8-OH(d)G immunoreactivity in mouse cerebellum from *Glul*$^{fl/fl}$ (*Left*) and *Glul*$^{fl/fl}$ × *Alb-Cre*$^{+}$ (*Right*) mice is presented in the absence (*Lower*) or presence (*Upper*) of costaining with calbindin (green) and Hoechst 34580 (blue). One representative of $n = 3$ is demonstrated. (Scale bar: 50 μm.)

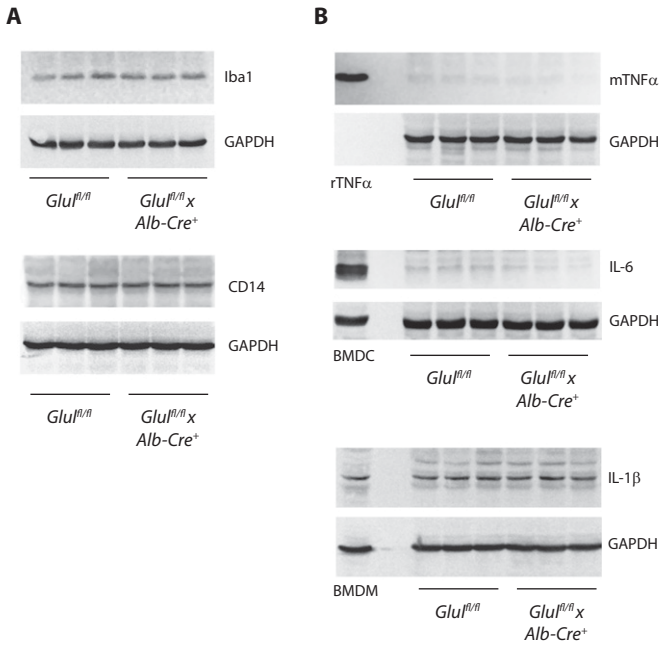

Fig. S5. Microglia activation marker and proinflammatory cytokine protein expression in the cerebral cortex of WT and *Glul*$^{fl/fl}$ × *Alb-Cre*$^{+}$ mice. Detection of microglia activation markers Iba1 and CD14 (*A*) or proinflammatory cytokines IL-1β, IL-6, or TNF-α (*B*) protein levels in mouse cerebral cortex by Western blot analysis is shown. Recombinant mouse TNF-α (*Upper*, rTNF-α), bone marrow-derived dendritic cells [LPS (30 ng/mL, 3 h)-treated] (*Middle*, BMDC), or bone marrow-derived mouse macrophages [LPS (10 ng/mL, 24 h)-treated] (*Lower*, BMDM) was used as a positive control. GAPDH served as a loading control ($n = 3$ for *Glul*$^{fl/fl}$ and $n = 5$ for *Glul*$^{fl/fl}$ × *Alb-Cre*$^{+}$ mice).

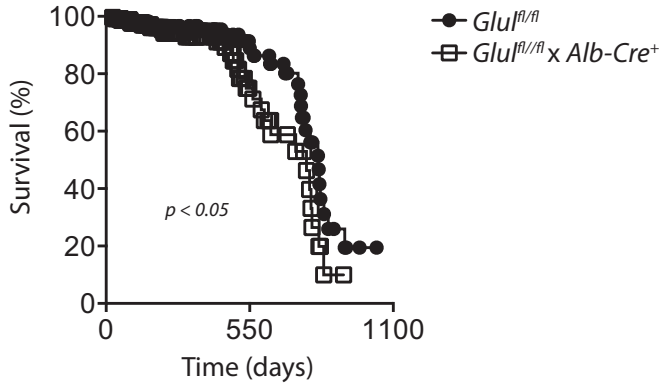

Fig. S6. *Glul*$^{fl/fl}$ × *Alb-Cre*$^{+}$ mice exhibit a slightly reduced life span compared with *Glul*$^{fl/fl}$ mice. A Kaplan–Meier curve of *Glul*$^{fl/fl}$ (●) and *Glul*$^{fl/fl}$ × *Alb-Cre*$^{+}$ (□) mice is shown over time (starting with $n = 246$–256; $P < 0.05$ using the Mantel–Cox test).