Supporting Information

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SI Materials and Methods

Global Cerebral Ischemia. Five minutes of tGCI were induced by bilateral common carotid artery occlusion combined with hypotension, using a method described previously (1) with some modifications (2). Male Sprague-Dawley rats (280–350 g) were anesthetized with 2.0% isoflurane in 70% nitrous oxide and 30% oxygen using a face mask. The rectal temperature was controlled at 37°C during surgery with a homeothermic blanket. The femoral artery was exposed and catheterized with a PE-50 catheter to allow continuous recording of arterial blood pressure. A midline neck skin incision was made, and the right jugular vein and both common carotid arteries were exposed. After i.v. injection of 150 IU/kg heparin, blood was quickly withdrawn via the jugular vein. When the mean arterial blood pressure became 30 mm Hg, both common carotid arteries were clamped with surgical clips. Blood pressure was maintained at 30 mm Hg by withdrawing or infusing blood through the jugular vein during the ischemic period. After 5 min of ischemia, the clips were removed, and the blood was reinfused. Regional cerebral blood flow was monitored using laser Doppler flowmetry as previously described (3). Sham-operated animals underwent exposure of vessels without blood withdrawal or clamping of carotid arteries. The animals were maintained in an air-conditioned room at 20°C with *ad libitum* access to food and water before and after surgery.

siRNA Administration. Transfection of siRNAs into animal cells results in potent, long-lasting, posttranscriptional silencing of specific genes (4, 5). Recently, several groups succeeded in the knockdown of target proteins by inducing siRNAs into the central nervous system *in vivo* (6–9). Decreasing PIDD expression with siRNA abrogated time-dependent caspase-2 activation *in vitro* (10). These studies inspired us to use siRNA to reveal the roles of PIDD.

We used four different siRNAs: FITC-siRNA, siSTABLE non-targeting control siRNA, predesigned ON-TARGET*plus* PIDD siRNA (PIDD-siRNA), and custom-made *in vivo* siSTABLE PIDD-siRNA. FITC-siRNA was purchased from Santa Cruz Biotechnology (sc-36869) to evaluate transfection efficiency. Other siRNAs were purchased from Dharmacon. Control siRNA (D-001700–01-05) was used as an off-site siRNA control. PIDD-siRNA and siSTABLE PIDD-siRNA were used to inhibit PIDD expression. PIDD-siRNA (L-085244–01) was a mixture of four siRNA-targeting PIDD sequences of 5′-ACCGUAAGCUUCAGCGUAUUU-3′, 5′-CGAUGUAGAUGCUGACCGUUU-3′, 5′-GCUGAAGAGGUGCGUGCCAUU-3′, and 5′-CCACAGCCUUGGACCGUGAUU-3′. siSTABLE PIDD-siRNA was an siRNA targeting the PIDD sequence of 5′-AGACCUACCUGCCGAGCUAUU-3′ with *in vivo* siSTABLE option, which chemically modifies the enhancing stability of siRNAs even under non-RNase-free conditions. The modifications are typically on the backbone or at the 2′-position of the sugar. ON-TARGET*plus* and siSTABLE option contains the modification against 3′-untranslated regions (11). These positions are likely sites for degradation mediated by nucleases. The transfection agent was 10 mM of jetSI (403–05; Polyplus Transfection) because it had been used previously for effective rodent brain delivery of siRNA (7). JetSI was used with 20 mM of dioleoylphosphatidylethanolamine (P1223; Sigma-Aldrich) at a 1:2 molar ratio. JetSI and dioleoylphosphatidylethanolamine were used at a nitrogen/phosphate ratio of 1.8. Transfection mixes containing 0.2 μg/μl of siRNA (for 1 μg siRNA injection) or 1 μg/μl of siRNA (for 5 μg siRNA injection) were prepared according to the manufacturer's protocol. For immunofluorescence, the transfection mix containing 1 μg of FITC-siRNA was injected intracerebroventricularly (bregma: 1.4 mm lateral, 0.8 mm posterior, 3.6 mm deep). Anesthetized animals were perfused with 10 U/ml heparin saline solution and subsequently with 4% formaldehyde in PBS solution 48 h after the FITC-siRNA injection to confirm transfection efficiency. The brains were removed, postfixed for 24 h, and sectioned at 50 μm with a vibratome. The sections were then covered with VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (H-1500; Vector Laboratories). For titration, the transfection mixes containing 5 μg of control siRNA, 0.2, 1, and 5 μg of predesigned PIDD-siRNA, and 0.2, 1, and 5 μg of siSTABLE PIDD-siRNA were injected intracerebroventricularly. Forty-eight hours after injection, the animals were killed and Western blotting was performed. For inhibition of PIDD after tGCI, we used 5 μg of control siRNA and siSTABLE PIDD-siRNA 48 h before tGCI. Animals were killed 8 h after tGCI, followed by a Western blot procedure.

Western Blot Analysis. Fresh brain tissue was removed after 1, 4, 8, 24, and 72 h of reperfusion. The tissue was cut into 1-mm coronal slices using a brain matrix (Zivic Laboratories), and the bilateral hippocampi were removed. Then, the hippocampal CA1 subregion, which is above the hippocampal fissure and CA3 subregion (i.e., the corner area), was quickly dissected under a microscope and used as a sample. Protein extraction of the cytosolic and mitochondrial fractions was performed using a multiple centrifugation method as described previously (12). The tissue was homogenized by gently douncing 30 times in a glass tissue grinder (357538; Wheaton) in 7 volumes of cold suspension buffer (20 mM Hepes-KOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, and 1 mM EGTA plus 0.7% protease and 1% phosphatase inhibitor cocktails [P8340, P2850, P5726; Sigma-Aldrich]). The homogenate was centrifuged at 750 × *g* for 10 min at 4°C and then at 10,000 × *g* for 20 min at 4°C. The 10,000 × *g* pellets were used to obtain the mitochondrial fraction. The supernatant was further centrifuged at 100,000 × *g* for 60 min at 4°C, and was then used for cytosolic analysis. For siRNA-treated animals, only the injection side of the hippocampal CA1 subregion was used as a sample. Whole hippocampal lysate was prepared with cell lysis buffer (9803; Cell Signaling Technology). Protein concentrations were determined by comparison with a known concentration of BSA using a kit (23225; Pierce). After adding a one-third volume of lithium dodecyl sulfate (LDS) sample buffer (NP0007; Invitrogen), we loaded equal amounts of the samples (5–10 μg) per lane. Jurkat cell lysate (sc-2204; Santa Cruz Biotechnology) was used as a positive control. Proteins were separated with the NuPAGE gel system (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Invitrogen). The primary antibodies were a 1:100 dilution of an anti-PIDD antibody (Santa Cruz Biotechnology), a 1:1,000 dilution of an anti-caspase-2L antibody (611023; BD Biosciences), a 1:1,000 dilution of an anti-RAIDD antibody (sc-7880; Santa Cruz Biotechnology), a 1:100 dilution of an anti-Bid antibody (sc-6538; Santa Cruz Biotechnology), a 1:1,000 dilution of an anti-Bax antibody (2772; Cell Signaling Technology), a 1:2,000 dilution of an anti-Bcl-X_L antibody (2764; Cell Signaling Technology), a 1:1,000 dilution of an anti-p53 antibody (554147; BD Biosciences), a 1:100,000 dilution of an anti-β-actin antibody (A5441; Sigma-Aldrich), and a 1:100,000

dilution of an anti-cytochrome oxidase subunit IV antibody (A21348; Invitrogen). Western blots were performed with horseradish peroxidase-conjugated anti-mouse IgG (7076; Cell Signaling Technology), anti-rabbit IgG (7074; Cell Signaling Technology), or anti-goat IgG (AP106P; Millipore) using SuperSignal West Pico and/or Femto substrate (1856135, #1856189; Pierce). Images were scanned with a GS-700 imaging densitometer (Bio-Rad Laboratories) and the results were quantified using MultiAnalyst software (Bio-Rad). To confirm the specificity of the antibody, Western blotting was performed with the anti-PIDD antibody neutralized with a fivefold excess of blocking peptide (sc-32161 P; Santa Cruz Biotechnology) overnight at 4°C.

Co-IP. A sample of the cytosolic fractions was prepared as described in the Western blotting method. The procedure for precipitation was performed as described previously (13). Fresh brain tissue was obtained from the hippocampal CA1 subregion after 1, 4, 8, and 24 h of reperfusion. Protein concentrations were determined by comparison with a known concentration of BSA using a kit (23225; Pierce). Fifty micrograms of protein from the cytosolic fraction were used for co-IP. Ramos cell lysate (sc-2216), PC-12 cell lysate (sc-2250), and Jurkat cell lysate (sc-2204; all from Santa Cruz Biotechnology) were included as positive controls. The protein sample was incubated with 50% slurry of protein G-Sepharose (17–0618-01; GE Healthcare) for 1 h at 4°C, and this mixed sample was centrifuged at 12,000 × *g* for 1 min. The supernatant was incubated with 1 μg of polyclonal rabbit anti-PIDD antibody (ALX-210–920-C050; Alexis Laboratories) or monoclonal rat anti-caspase-2 antibody (ALX-804–356-C100; Alexis Laboratories) and 20 μl of protein G-Sepharose (50% slurry) for 2 h at 4°C. The negative control was prepared with protein G-Sepharose without an antibody. The 12,000 × *g* pellets were washed three times and used as the samples bound to each antibody. After adding one-third volume of LDS sample buffer to the samples, they were boiled to remove the Sepharose beads. After centrifugation at 12,000 × *g* for 1 min, the supernatant was immunoblotted with a 1:100 dilution of an anti-PIDD antibody (sc-32161; Santa Cruz Biotechnology), a 1:1,000 dilution of an anti-RAIDD antibody (sc-7880; Santa Cruz Biotechnology), a 1:1,000 dilution of an anti-caspase-2L antibody (611023; BD Biosciences), or a 1:1,000 dilution of an anti-caspase-3 antibody (sc-7148; Santa Cruz Biotechnology).

Assay of Bid Cleavage. Fresh brain tissue was taken from the hippocampal CA1 subregion and homogenized with a Teflon homogenizer (357538; Wheaton) in 7 volumes of ice-cold lysis buffer (9803; Cell Signaling Technology). The homogenate was sonicated and centrifuged at 14,000 × *g* for 10 min at 4°C. The supernatant was collected and the protein concentration was determined by comparison with a known concentration of BSA using a kit (23225; Pierce). Forty micrograms of the sample were incubated in the caspase-2 reaction buffer (50 mM Hepes, pH 7.2, 50 mM NaCl, 0.1% Chaps, 10 mM, EDTA, 5% glycerol, and 10 mM DTT) for 1 h at 37°C with 1 or 3 units of recombinant activated caspase-2 (CC127; Millipore) or caspase-2 reaction buffer alone. The reactions were stopped by addition of LDS sample buffer, and a half volume of the products was immunoblotted with a 1:100 dilution of an anti-Bid antibody (sc-6538; Santa Cruz Biotechnology), a 1:1,000 dilution of an anti-Bax antibody (2772; Cell Signaling Technology), a 1:1,000 dilution of an anti-Bcl-X_L antibody (2764; Cell Signaling Technology), or a 1:100,000 dilution of an anti-β-actin antibody (A5441; Sigma-Aldrich).

Immunofluorescent Staining. The experimental animals were killed after 1, 8, 24, and 72 h of reperfusion. The brains were removed, rapidly frozen in −50°C dry ice and stored at −80°C. They were sectioned with a cryostat to a thickness of 12 μm. Frozen brain sections at the level of the hippocampus were placed on slides and fixed by 4% paraformaldehyde in PBS solution, pH 7.4, for 15 min. Nonspecific binding was blocked by 3% BSA. To evaluate co-localization of PIDD and NeuN, RAIDD and NeuN, or procaspase-2/caspase-2 and NeuN, we performed double immunofluorescent staining. For double immunofluorescent staining of PIDD and NeuN, the sections were immunostained with a 1:50 dilution of an anti-PIDD antibody (sc-32161; Santa Cruz Biotechnology), followed by Alexa 594-conjugated anti-goat IgG (A11058; Invitrogen). The sections were then incubated with a 1:500 dilution of an Alexa 488-conjugated anti-NeuN antibody (MAB377X; Millipore). For double immunofluorescent staining of RAIDD and NeuN, the sections were immunostained with a 1:200 dilution of an anti-RAIDD antibody (sc-7880; Santa Cruz Biotechnology), followed by Alexa 594-conjugated anti-rabbit IgG (A21207; Invitrogen). The sections were then incubated with a 1:500 dilution of an Alexa 488-conjugated anti-NeuN antibody (MAB377X; Millipore). For double immunofluorescent staining of procaspase-2/caspase-2 and NeuN, the sections were immunostained with a 1:500 dilution of anti-NeuN antibody (MAB377X; Millipore), followed by FITC-conjugated anti-mouse monovalent Fab fragments of a secondary antibody (715–097-003; Jackson ImmunoResearch Laboratories) at a dilution of 1:100 for labeling and blocking of NeuN. The sections were then incubated with a 1:100 dilution of an anti-caspase-2L antibody (611023; BD Biosciences), followed by Alexa 594-conjugated anti-mouse IgG (A21203; Invitrogen). The sections were then covered with VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (H-1500; Vector Laboratories). To evaluate co-localization of PIDD, RAIDD, and caspase-2, we performed triple immunofluorescent staining, for which the sections were incubated with an anti-PIDD antibody (sc-32161; Santa Cruz Biotechnology), followed by Alexa 594-conjugated anti-goat IgG (A11058; Invitrogen), as described earlier. Then, the sections were incubated with an anti-RAIDD antibody (sc-7880; Santa Cruz Biotechnology), followed by Alexa 488-conjugated anti-rabbit IgG (A21206; Invitrogen), as described earlier. The sections were then incubated with an anti-caspase-2L antibody (611023; BD Biosciences), followed by 7-amino-4-methylcoumarin-3-acetic acid-conjugated anti-mouse IgG antibody (CI-2000; Vector Laboratories). The sections were then covered with VECTASHIELD mounting medium (H-1400; Vector Laboratories) and were examined under an Axioplan 2 microscope (Carl Zeiss).

Histological Analysis of Hippocampal Injury and *In Situ* Labeling of DNA Fragmentation. Anesthetized animals were perfused with 10 U/ml heparin saline solution and subsequently with 4% formaldehyde in PBS solution after 1, 8, 24, and 72 h of reperfusion. The brains were removed, postfixed for 24 h, and sectioned at 50 μm with a vibratome. For histological assessment, the sections were stained with cresyl violet. For *in situ* labeling of DNA fragmentation, brain sections at the level of the hippocampus were placed on slides and stained using TUNEL reaction to detect the DNA-free 5′-OH ends as described previously (14), with some modification. Briefly, the sections were incubated with NeuroPore (4820–30-01; Trevigen) for 30 min. They were placed in 1× terminal deoxynucleotidyl transferase buffer (16314–015; Invitrogen) for 5 min, followed by reaction with terminal deoxynucleotidyl transferase enzyme (10533–073; Invitrogen) and biotinylated 16-dUTP (1093070; Roche Diagnostics) at 37°C for 60 min. The sections were washed in 2 × 300 mM sodium chloride and 30 mM sodium citrate (pH 7.4) for 15 min, followed by washing in PBS solution three times for 2 min each time. The avidin-biotin technique was applied, and nuclei were counterstained with hematoxylin solution.

Cell Death Assay. To quantify apoptotic-related DNA fragmentation, a commercial enzyme immunoassay was used to determine cytoplasmic histone-associated DNA fragments (1774425; Roche Molecular Biochemicals), which detect apoptotic but not necrotic cell death. A sample was prepared as described in the Western blotting method. The protein concentration of the cytosolic fraction was determined. A cytosolic volume containing 20 μg of protein was used for the ELISA, following the manufacturer's protocol. Briefly, the cytosolic sample was placed onto a streptavidin-coated microplate and was incubated with anti-histone-biotin and anti-DNA-peroxidase, both of which bind to the nucleosomes, for 2 h at room temperature. After washing unbound components, the amount of nucleosomes was quantitatively determined by the peroxidase retained in the immunocomplex with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) solution at 405 nm.

Cell-Counting Procedure. For quantification of the TUNEL studies, intact pyramidal cells with a distinct nucleus or TUNEL-positive cells were counted in a 1-mm length of the middle portion of the CA1 subfield by two blinded counters, as described previously (15).

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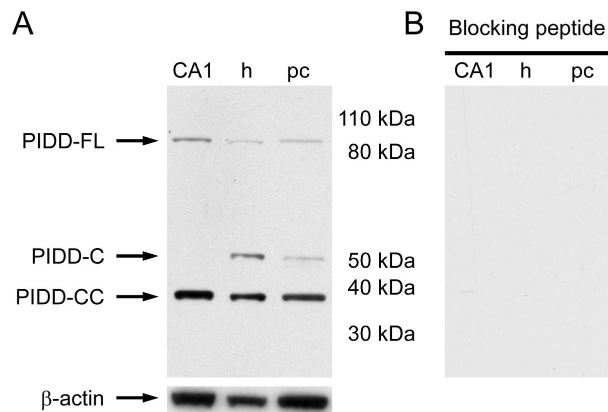

Fig. S1. Confirmation of the specificity of the anti-PIDD antibody. (*A*) Western blot analysis of PIDD using the cytosolic fraction of the hippocampal CA1 subregion, the rat hippocampal lysate, and the positive control. PIDD-FL, PIDD-C, and PIDD-CC were detectable at ≈100, 53, and 37 kDa in the rat hippocampal lysate and the positive control. In the cytosolic fraction of the hippocampal CA1 subregion, PIDD-FL and PIDD-CC were detectable, but PIDD-C was not detected. CA1, cytosolic fraction of the hippocampal CA1 subregion; h, whole cell extract of the rat hippocampus; pc, Jurkat cell lysate as a positive control. (*B*) Western blot analysis of PIDD using the cytosolic fraction of the hippocampal CA1 subregion, the rat hippocampal lysate, and the positive control with blocking peptide. An anti-PIDD antibody was neutralized with a fivefold excess of blocking peptide overnight at 4°C, and Western blot analysis was performed. No PIDD bands were detected with the blocking peptide.

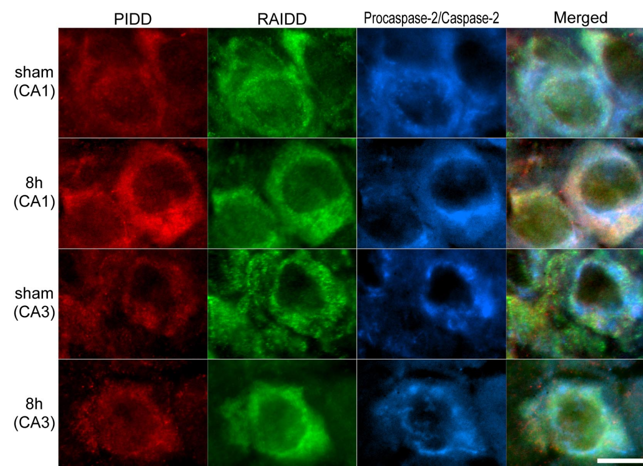

Fig. S2. Co-localization among PIDD, RAIDD, and procaspase-2/caspase-2 in the hippocampal CA1 subregion after tGCI. Fluorescent triple staining of PIDD (red), RAIDD (green), and procaspase-2/caspase-2 (blue) showed that PIDD increased and co-localized with RAIDD and procaspase-2/caspase-2 in the cytosol 8 h after tGCI. No co-localization was seen in the CA1 subregion of the shams or the CA3 subregion. Scale bar, 10 μm.

Fig. S3. Titration of PIDD-siRNA. Control siRNA, predesigned PIDD-siRNA, and siSTABLE PIDD-siRNA were administered intracerebroventricularly. siRNA injection dosages were 0.2, 1, and 5 μg. The animals were killed 48 h after the siRNA injection, and Western blotting was performed, which showed that PIDD-FL and PIDD-CC were significantly down-regulated in the 5-μg siSTABLE PIDD-siRNA-treated animals compared with the non-treated or control siRNA-treated animals (*$P < 0.05$). RAIDD or p53, which are related proteins, did not decrease with siRNA treatment. There were no significant differences in PIDD-FL, PIDD-CC, RAIDD, and p53 expression between the non-treated and control siRNA-treated animals. β-actin was used as an internal control. NT, non-treated; c, control siRNA-treated; Predesigned, predesigned PIDD-siRNA-treated; siSTABLE, siSTABLE PIDD-siRNA-treated.

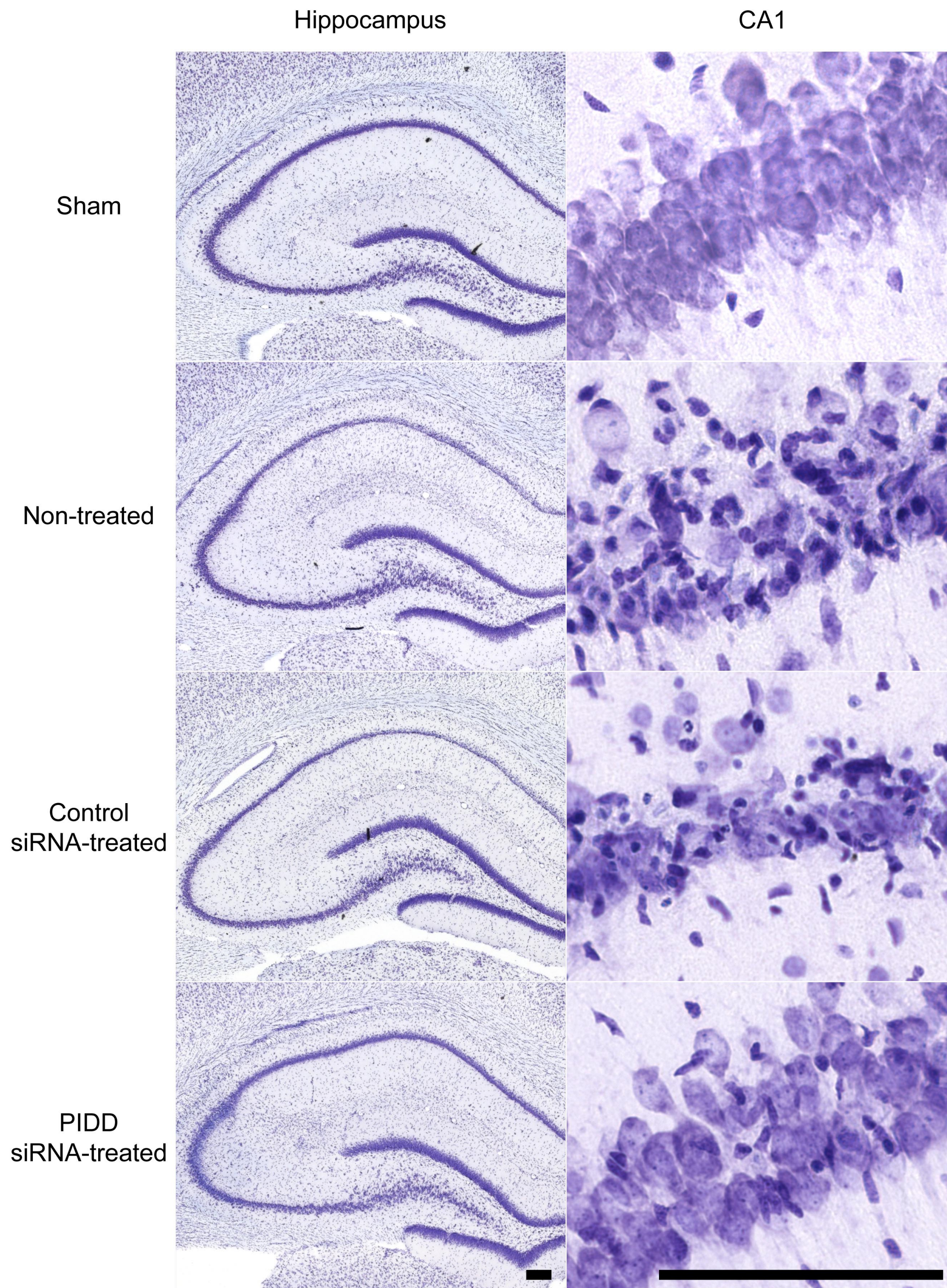

Fig. S4. Cresyl violet staining 7 days after tGCI. In the shams, no hippocampal CA1 damage was observed. Seven days after tGCI, most CA1 neurons in the non-treated and control siRNA-treated animals were damaged; they had shrunken, condensed nuclei. However, the normal features of the nuclei of many neurons were well preserved in the PIDD-siRNA-treated animals, although sparse neuronal damage was observed in the hippocampal CA1 subregion. Scale bars, 100 μm.