Supporting Information

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SI Materials and Methods

Real-Time PCR. Total RNA was isolated using the miRNeasy kit following manufacturer instructions (Qiagen). A total of 50 ng of total RNA was used for reverse transcription reaction using the Taqman miR-223 and U6 snRNA reverse transcription primers. MicroRNA quantitative real-time PCR was done using the Taqman real-time probes and an Applied Biosystems ABI 7900 Sequence Detection system. U6 snRNA level was used to normalize miR-223 values. Each sample was run at least three independent times.

Gene Ontology Analysis. Gene functional annotation clustering was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) v6.7 using the total set of TargetScan (1) or miRanda (2) predicted targets for Mus musculus miR-223 (mmu-miR-223). The mouse genome was used as a genetic background. *P* values represent a modified Fisher's exact test (EASE score) (3, 4).

Western Blot Analysis. Hippocampal tissue lysates were generated in RIPA buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS using a Dounce homogenizer. Proteins were size separated using standard Western blot analysis. Immunoblotting was performed using the following primary antibodies; mouse anti-GluR2, mouse anti-NR2B, mouse anti-mGluR1/5 [University of California Davis/National Institutes of Health (NIH) NeuroMab facility], rabbit anti-NR1 (Sigma-Aldrich), rabbit anti–PSD-95 (Millipore), and goat antiactin HRP (Santa Cruz Biotechnology). NIH ImageJ quantification of Western blots band intensities was normalized to actin protein levels.

Dual-Luciferase Assay. The 3′-UTR regions of interest were cloned into the Promega psiCHECK-2 dual-luciferase reporter construct. MiR-223 was stably expressed in 293 cells using a retrovirus expressing the genomic fragment of miR-223. The cells were transfected with constructs indicated in the figures (Fig. 2B–E and Fig. S3G) using Fugene HD transfection reagent following manufacturer instructions (Roche Applied Science). After 48 h, a luciferase assay was performed using the dual-luciferase reporter assay system following the manufacturer's instructions (Promega).

Electrophysiological Recording. Horizontal hippocampal slices (300 μm) were prepared from mice (postnatal day 15–20) using standard methods. Whole cell patch-clamp experiments were performed at room temperature (25 °C) with an EPC-10 amplifier (HEKA Elektronik). The extracellular solution was artificial cerebrospinal fluid (ACSF) containing (in millimoles): NaCl 125, KCl 2.5, $KH_2PO_4$1.3, $NaHCO_3$ 25, $CaCl_2$, $MgCl_2$ 1.3, Na ascorbate 1.3, Na pyruvate 0.6, dextrose 10, saturated with 95% O_2 5% CO_2 (pH 7.4). Patch pipettes (7 MΩ) were filled with 110 mM $CsCH_3SO_3$, 30 mM CsCl, 8 mM NaCl, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM ATP, 0.05 mM EGTA, pH 7.3). Cell membrane potential was held at −60 mV. For spontaneous miniature excitatory postsynaptic current (mEPSC) recording, extracellular solution also contain picrotoxin (100 μM) and TTX (0.5 μM). Primary hippocampal neuron recordings were conducted using the same conditions described above including 10 μM (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d] cyclohepten-5,10-imine (MK801) to measure AMPA receptor currents. For subunit-selective inhibitor experiments, either 10 μM philanthotoxin-433 (PhTX-433) or 3 μM ifenprodil were perfused into recording media following 3 min of mEPSC recording. Following perfusion, 15 min were allowed for pharmacological inhibition to take effect and mEPSCs were recorded for an additional 3 min. Rectification experiments were conducted on primary neurons at DIV12–14 using perforated-patch clamp recording for −80 to +40 mV, allowing 1 min of recording at each voltage. The pipette solution 120 mM KCl with gramicidin D (50 μg/mL) was used. Recording data were analyzed with Minianalysis-6 (Synaptosoft) and OriginPro8.1 software.

Fluo-4 Calcium Mobilization Assay. Confocal laser scanning microscope Carl Zeiss LSM 510 was used to monitor the Fluo-4 (Invitrogen) calcium indicator dye in real time. Plan-Apochromat 40×/1.3 oil objective was used for live imaging at 37 °C. Carl Zeiss LSM 5 software was used for image acquisition. Changes in free intracellular calcium levels were recorded following 100 μM NMDA stimulation in primary mouse hippocampal neurons. Briefly, neurons cultured on polyornithine-coated glass coverslips were loaded with Fluo-4 dye that was added to the culture media for 30 min at 1 μM final concentration. After one wash with 37 °C HBSS + 2 mM calcium chloride, coverslips were placed in a 37 °C heated adapter on the confocal microscope in the same buffer. Time series images were taken every 10 s starting with 2 min to establish a baseline and then the buffer was replaced with 100 μM NMDA in HBSS + 2 mM calcium chloride + 10 μM glycine. Areas of increased fluorescence following NMDA stimulation were quantified using LSM 510 software and plotted against time to produce an XY time series plot.

Transient Global Ischemia Model. The Animal Care and Use Committee of Johns Hopkins Medical Institutions approved experimental protocols for animals. *MiR-223* knockout mice were a kind gift from Fernando Camargo (Harvard University Department of Stem Cell and Regenerative Biology, Boston, MA) (5). Male mice (age: 8–12 wk) were anesthetized with 2–3% (vol/vol) isoflurane for induction and 1–1.5% for maintenance. To maintain temperature, a thermostatically controlled heating pad adjusted at 37.5 °C was used. Transient global ischemia was produced by performing bilateral common carotid artery occlusion (BCCAO) using a curved bulldog artery clamp. The mice were kept under BCCAO for 20 min followed by reperfusion. The mice were kept on a 37.5 °C pad until they recovered from anesthesia, then they were maintained at 25 °C. A consistent 70–75% decrease in blood flow was observed over the forebrain using laser doppler flowmetry. Arterial blood pH, PO_2, glucose, hemoglobin, and hematocrit were monitored and did not show a difference between groups. At 72 h after BCCAO, a fear conditioning memory test was conducted. Six days after BCCAO, mice were perfused with PBS followed by 4% paraformaldehyde under sodium pentobarbital anesthesia. The brains were removed for tissue processing.

Brain Tissue Processing and Lesion Volume Measurement. Brains were fixed with 4% paraformaldehyde overnight at 4 °C and then cryoprotected in 30% sucrose for 24 h at 4 °C. Tissue was sectioned on a freezing stage sliding microtome into a series of 30-μm serial sections, and stained every sixth section with Nissl stain, imaged, and quantified using the NIH ImageJ software. Lesion volume was calculated using the formula: lesion volume (mm^3) = (sum of unstained serial surface areas) × 0.03 × 6.

Nissl and Immunostaining. Tissue sections were mounted on slides, dried overnight, defatted in alcohol, rehydrated in series of decreasing alcohol concentrations, followed by water, and then stained in 0.1% thionin acetate. After differentiation in series

of increasing alcohol concentrations, slides were cleared in xylene and then mounted with distyrene-plasticizer-xylene (DPX) and covered with glass coverslips. Images were acquired using Carl Zeiss Axioplan2 microscope, Plan Neofluar 5×/0.15 DIC objective, and a Carl Zeiss AxiocamHRC camera at room temperature with Axiovision 4.8.2 software. Immunostaining for the neuronal nuclei marker NeuN and GFAP were performed in free-floating sections. Sections were permeabilized in 0.3% TX-100 in PBS for 20 min and then blocked in 5% normal goat serum. Images were acquired using confocal laser scanning microscope Carl Zeiss LSM 710, Plan-Apochromat 40×/1.4 oil objective at room temperature. Carl Zeiss Zen software was used to acquire tile scan and stitch tiles to cover the hippocampus.

Fear Conditioning. As illustrated in Fig. S5*C*, on day 1 (training) mice were placed individually in a fear conditioning (FC) apparatus with a metal grid floor (Med Associates). After a 2.5-min baseline period, 1 tone-shock pairing was presented. The pairing consisted of a 30-s 85 dB 2,000-Hz tone (cue) simultaneously ending with a 2-s 0.5-mA shock. The mice remained in the chamber for 2 min after the shock and then were returned to their cages. The total duration of training was 300 s. Freezing (immobility) was traced and analyzed using a video-based tracking system. On day 2 (contextual FC) mice were placed back individually in the same fear conditioning chamber for 300 s with no tones or shocks and their activity/freezing was recorded. On day 3 (cued FC) mice were placed in a novel open top white wooden cube that was placed in turn inside the test chamber. After 1 min, the 30-s tone was presented repeatedly for 240 s with 30-s intervals. Activity/freezing behavior was recorded.

Primary Neuronal Cultures. Mouse primary hippocampal neuronal cultures were prepared from gestational day 18 fetal or postnatal day 1 CD-1 mice. Briefly, the time pregnant mouse pups were killed by decapitation and then the uterine horns removed in a sterile dish. The cortices were dissected, meninges were removed and then hippocampi were separated from the cortex. After 5-min incubation in 0.027% trypsin–EDTA solution, the cells were mechanically dissociated in modified Eagle's medium (MEM). The neurons were plated on polyornithine precoated plates or glass coverslips. For excitotoxic studies, neurons were seeded in MEM, 10% horse serum, 25 mM glucose, and 2 mM L-glutamine. For calcium influx studies, neurons were seeded in neurobasal, 1% FBS, 2% B-27 supplement, and 2 mM L-glutamine. Neurons were maintained in a 7% CO_2 humidified 37 °C incubator. The neuronal cultures were inhibited with 5-fluoro-2′-deoxyuridine after 2 d to inhibit proliferation of nonneuronal cells.

Excitotoxicity. Primary hippocampal neurons were exposed to 500 μM NMDA plus 10 μM glycine for 5 min, and replaced with MEM, 10% horse serum, 25 mM glucose, and 2 mM L-glutamine to elicit cell death. The cultures were stained 24–48 h later with 1 μg/mL Hoescht 33342, which stains all cell nuclei and 7 μM propidium iodide, which stains dead cell nuclei. Cells are imaged and counted with unbiased computer-assisted cell counting (Axiovision 4.8 software; Carl Zeiss). Images were acquired using Carl Zeiss Axiovert 200M microscope, EC-Plan Neofluar 20×/0.50 objective, Carl Zeiss AxiocamMRC camera at room temperature. Glial nuclei are bigger in size and fluoresce at a different intensity than neuronal nuclei. Using this criteria, glia were gated out. Percent cell survival is determined as the ratio of (total minus dead) cells to total cells.

MiR-223 Lentiviral Constructs. The microRNA lentiviral expression vector contains essential packaging elements, central polypurine tract (CPPT), woodchuck posttranscriptional regulatory element (WPRE), and EGFP driven by the human ubiquitin C promoter. An approximately 600-bp fragment including the genomic miR-223 locus driven by the U6 promoter was cloned into that vector. A nontargeting miRNA (Open Biosystems) was cloned into the same vector and used as a control. Anti–miR-223 sponge: A 7-tandem repeat of an artificial bulged miR-223 binding site (5′ GGGGTATTTTAGAACTGACA 3′) and a 7-tandem repeat control mismatch sequence (5′ GGGGTGCTTTTAGAACC-AGCA 3′) were cloned into the lentiviral expression vector.

Adeno-Associated Viral Vectors. An approximately 600-bp fragment including the genomic miR-223 locus driven by the U6 promoter was cloned into adeno-associated viral (AAV)2 viral vector obtained from the University of Iowa (Iowa City, Iowa) gene transfer vector core (UI-GTVC). A nontargeting miRNA (Open Biosystems) was cloned into the same vector and used as a control. The AAV was produced by the UI-GTVC. AAV2 was desalted by dialysis against HyClone formulation buffer 18 before injection into the brain.

Stereotaxic Injection. C57BL/6, *miR-223* WT or KO mice (8–10 wk of age) were anesthetized with 50 mg/kg pentobarbital via i.p. injection. A midline incision in the skin over the skull was made and the entire superior part of the skull was exposed. Each mouse head was immobilized in a stereotaxic apparatus (David Kopf Instruments). Bilateral burr holes were made using the following coordinates: from bregma rostral, −0.5 mm; lateral, ±2.0 mm. NMDA (20 nmols in 0.2 μL) were injected into the striatum at ventral, −4.0 mm from bregma. For AAV vector injection, 4 μL of nontargeting miRNA-encoding virus (NT-AAV) was injected in the left striatum and a similar volume of miR-223–AAV injected in the right striatum 1 wk before NMDA injection. The injections were made over 5 min using a 10-μL Hamilton syringe. The needle was left in place for an additional 5 min after injection. Mice were killed as described above, 48 h after injection. Coordinates used for AAV injection in the hippocampal cornu ammonis 1 (CA1) area: from bregma caudal, 2.0 mm; lateral, ±1.6 mm; and 1.5 mm ventral. The injections (1 μL AAV–miR-223 or AAV-NT) were made over 5 min using a 1-μL Hamilton syringe. The needle was left in place for an additional 2 min after injection.

Statistical Analysis. Unless otherwise indicated, statistical analysis was performed using Prism software (GraphPad). Statistical tests were used with alpha level at 0.05. Two group comparisons were evaluated using two-tailed *t* test. Multiple group comparisons were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post hoc test. Data were expressed as mean ± SEM. Experiments for quantification were performed in a blinded fashion.

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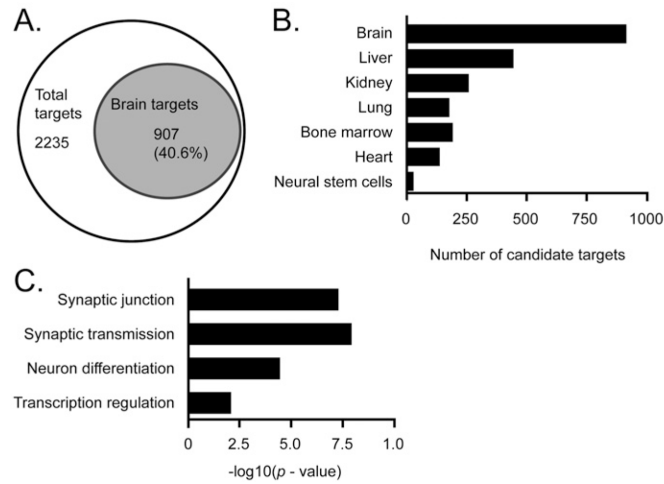

Fig. S1. Functional annotation of miR-223 potential CNS targets predicted by miRanda. DAVID bioinformatic (v6.7) analysis of miRanda (August 2010 release) predicted miR-223 targets. (*A*) Venn diagram of miR-223 predicted targets showing brain expressed subset. (*B*) Select tissue distribution comparison for miR-223 predicted targets (see Dataset S2 for full comparison). (*C*) Functional annotation analysis for brain-expressed miR-223 predicted targets. The *x* axis represents the EASE score *P* values for gene-enrichment annotation clusters.

Fig. S2. MiR-223 targets GluR2 and NR2B. (*A*) ClustalW multiple sequence alignment shows conservation of miR-223 predicted target site in GluR2 3′-UTR among mammals (*, indicates conservation of nucleotide across indicated species). (*B*) ClustalW multiple sequence alignment shows conservation of miR-223 predicted target site in NR2B 3′-UTR between mouse and rat and in NR3A among human, chimpanzee, and rhesus monkey (*, indicates conservation of nucleotide across indicated species). (*C*) RNA hybrid algorithm predicted hybridization of miR-223 (*Upper*/green) with GluR2 mRNA 3′-UTR (*Lower*/red). Minimum free energy (mfe) required for hybridization is indicated. (*D*) RNAhybrid algorithm predicted hybridization of miR-223 (*Right*/green) with NR2B mRNA 3′-UTR (*Left*/red). Mfe required for hybridization is indicated. (*E*) Distribution of mEPSC amplitude from WT and *miR-223* KO mice hippocampal slices. (*F*) Current–voltage (*I–V*) plots using perforated-patch recording of AMPAR-mEPSCs of miR-223 WT ($n = 4$) and KO ($n = 5$) primary hippocampal neurons recorded at different holding potentials (HP, from −60 to 60 mV). Traces show means ± SEM. Note the inward rectification in miR-223 KO neurons, a signature of calcium-permeable AMPARs.

Fig. S3. Fluo-4 calcium mobilization assays in hippocampal neurons. (*A*) Confocal images of Fluo-4 loaded hippocampal neurons before (0 min) and after (5 min) 100 μM NMDA stimulation. (*B*) Quantification of regions of interest demonstrating effect of (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine (MK801) and 6,7-Dinitroquinoxaline-2,3(1H,4H)-dione (DNQX) on increased fluorescence over time in response to NMDA administered at 140 s. Data represent mean ± SEM. (Control) (n = 8), MK801 (n = 3), DNQX (n = 3). (*C*) Confocal images of Fluo-4–loaded postnatal hippocampal neurons isolated from *miR-223* WT (n = 8) or *miR-223* KO (n = 10) littermates, before (0 min) and after (2.5 min) 100 μM NMDA stimulation. (*D* and *E*) Quantification of regions of interest demonstrating effect of miR-223 on increased fluorescence over time in response to NMDA administered at 60 s. Data represent mean ± SEM. *$P < 0.05$ one-tailed t test. (*F*) Primary mouse hippocampal neurons were treated with HBSS or 100 μM NMDA for 10 min. (*Left*) Hoechst 33342 (blue)/propidium iodide (red) double staining for neurons. Propidium iodide-positive cells (red) are considered dead. (*Right*) Quantification of cell death in hippocampal neurons. (*G*) Dual-luciferase assay for GluR2 target site in the presence or absence of miR-223 sponge. HEK 293 cells stably expressing miR-223 were transfected with dual-luciferase reporter constructs expressing miR-223 target site in GluR2. Luciferase assay graph show Renilla/Firefly luciferase activity measured 48 h posttransfection. Means ± SEM of representative experiments performed in four replicates are shown.

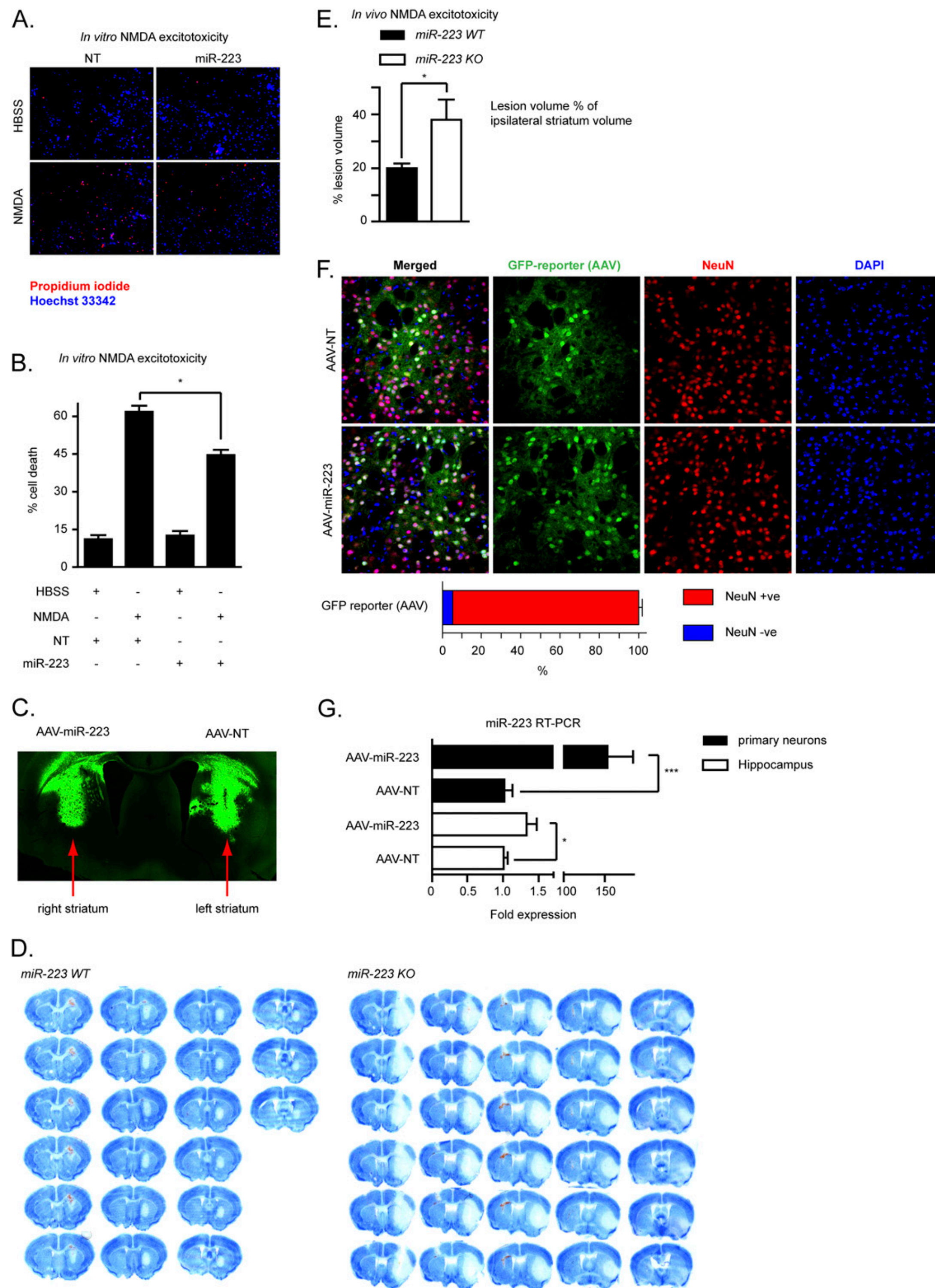

Fig. S4. MiR-223 regulates excitotoxic neuronal death. (*A*) Primary mouse hippocampal neurons were transduced with lentivirus overexpressing a non-targeting miRNA (NT) or miR-223. Hoechst 33342 (blue)/propidium iodide (red) double staining for neurons treated with HBSS or 500 μM NMDA. Propidium iodide-positive cells (red) are considered dead. (*B*) Quantification of the NMDA-induced cell death in hippocampal neurons. Neurons that overexpress miR-223 are protected against NMDA toxicity compared with NT overexpressing neurons. Data are the mean ± SEM (one-way ANOVA, $^*P < 0.05$ Tukey's multiple comparison post hoc test). NT-HBSS ($n = 10$), miR-223–HBSS ($n = 8$), NT-NMDA ($n = 12$), miR-223-NMDA ($n = 12$). (*C*) Green fluorescent protein (GFP) reporter expression in the striatum injected with AAV–miR-223 or AAV-NT. (*D*) Nissl stain demonstrating damage in striatum following NMDA injection as indicated by reduced staining areas. (*E*) Striatum lesion volumes percentage of total striatum volume. Means ± SEM are shown ($^*P < 0.05$, two-tailed *t* test). WT ($n = 5$), KO ($n = 4$). (*F*) AAV expression in the striatum. GFP reporter expression in the striatum injected with AAV–miR-223 (*Right*) or AAV-NT (*Left*). Colocalization of GFP-reporter (AAV) with the neuronal marker NeuN (red) and the general nuclear marker DAPI (blue) is shown (*Left*). *Right* panel demonstrates quantification of NeuN positive versus negative cells percentage of GFP-expressing cells ($n = 5$). (*G*) MiR-223 RT-PCR in primary hippocampal neurons and hippocampal tissue 5 d after AAV-mediated overexpression ($n = 4$). ($^*P < 0.05$, $^{***}P = 0.001$, one-tailed *t* test).

Fig. S5. Delayed CA1 neuronal cell death. (*A*) Immunostaining for antineuronal nuclei marker (anti-NeuN) showing neuronal loss in the CA1 hippocampal region following BCCAO in *miR-223* WT versus KO mice. (*B*) Green fluorescent protein (GFP) reporter expression in the CA1 area (arrowheads) of the hippocampus injected with AAV–miR-223 or AAV-NT. (*C*) Schematic diagram of fear conditioning memory test protocol. (*D*) Pain threshold analysis for *miR-223* WT versus KO mice. Mice were exposed to the indicated electrical shock currents and their response was recorded. There is no apparent difference between both genotypes in pain threshold. (*E* and *F*) Correlation between contextual memory freezing time and CA1 lesion volume in all mice that underwent transient global ischemia. R^2 value indicates the goodness of trend line fit for the data set ($P = 0.0005$, linear regression analysis). (*G*) Contextual fear conditioning test in AAV-NT and AAV–miR-223 groups. (*H*) Cued fear conditioning test in AAV-NT and AAV–miR-223 groups. Data points represent mean ± SEM. AAV-NT sham ($n = 9$), AAV-NT BCCAO ($n = 7$), AAV–miR-223 sham ($n = 9$), AAV–miR-223 BCCAO ($n = 8$). (*I*) AAV expression in the CA1 region of the hippocampus. GFP reporter expression in the CA1 region injected with AAV–miR-223 (*Right*) or AAV-NT (*Left*). Colocalization of GFP-reporter (AAV) with the neuronal marker NeuN (red) and the general nuclear marker DAPI (blue) is shown (*Left*). (*Right*) Quantification of NeuN positive versus negative cells percentage of GFP-expressing cells ($n = 5$).

Dataset S1. DAVID functional annotation of brain-expressed sublist of TargetScan miR-223 predicted targets

Dataset S1

TargetScan total predicted miR-223 targets, target tissue expression, and functional annotation of brain-expressed targets.

Dataset S2. DAVID functional annotation of brain-expressed sublist of miRanda miR-223 predicted targets

Dataset S2

miRanda total predicted miR-223 targets have similar target tissue expression distribution and functional annotation of brain-expressed targets as the TargetScan predicted list.