Journal of the American Heart Association

ORIGINAL RESEARCH

Stroke Causes DNA Methylation at *Ncx1* Heart Promoter in the Brain Via DNMT1/MeCP2/REST Epigenetic Complex

Natascia Guida, PhD; Angelo Serani, PhD; Luca Sanguigno, PhD Student; Luigi Mascolo, PhD; Ornella Cuomo, PhD; Salvatore Fioriniello, PhD; Domenico Marano, PhD; Floriana Della Ragione, PhD; Serenella Anzilotti, PhD; Paola Brancaccio, PhD; Pasquale Molinaro, PhD; Giuseppe Pignataro, MD, PhD; Lucio Annunziato, MD; Luigi Formisano, MD, PhD

BACKGROUND: REST (Repressor-Element 1 [RE1]-silencing transcription factor) inhibits Na^+/Ca^{2+}exchanger-1 (*Ncx1*) transcription in neurons through the binding of RE1 site on brain promoter *(Br)* after stroke. We identified a new putative RE1 site in *Ncx1* heart promoter *(Ht)* sequence (*Ht*-RE1) that participates in neuronal *Ncx1* transcription. Because REST recruits DNA-methyltransferase-1 (DNMT1) and MeCP2 (methyl-CpG binding protein 2) on different neuronal genes, we investigated the role of this complex in *Ncx1* transcriptional regulation after stroke.

METHODS AND RESULTS: Luciferase experiments performed in SH-SY5Y cells demonstrated that *Br* activity was selectively decreased by REST, whereas *Ht* activity was reduced by DNMT1, MeCP2, and REST. Notably, site-direct mutagenesis of *Ht*-RE1 prevented REST-dependent downregulation of *Ncx1*. Furthermore, in temporoparietal cortex of 8-week-old male wild-type mice (C57BL/6) subjected to transient middle cerebral artery occlusion, DNMT1, MeCP2, and REST binding to *Ht* promoter was increased, with a consequent DNA promoter hypermethylation. Intracerebroventricular injection of siREST prevented DNMT1/MeCP2 binding to *Ht* and *Ncx1* downregulation, thus causing a reduction in stroke-induced damage. Consistently, in cortical neurons subjected to oxygen and glucose deprivation plus reoxygenation *Ncx1* knockdown counteracted neuronal protection induced by the demethylating agent 5-azacytidine. For comparisons between 2 experimental groups, Student's *t* test was used, whereas for more than 2 experimental groups, 1-way ANOVA was used, followed by Tukey or Newman Keuls. Statistical significance was set at $P<0.05$.

CONCLUSIONS: If the results of this study are confirmed in humans, it could be asserted that DNMT1/MeCP2/REST complex disruption could be a new pharmacological strategy to reduce DNA methylation of *Ht* in the brain, ameliorating stroke damage.

Key Words: DNA methylation ■ DNMT1 ■ MeCP2 ■ NCX1 ■ REST ■ stroke

It is well established that the isoform 1 of NCX (sodium/calcium exchanger1), a plasma membrane protein regulating intracellular calcium and sodium homeostasis in the brain, exerts a neuroprotective role in stroke.[1,2] We previously demonstrated that stroke induces *Ncx1* downregulation at the transcriptional level via an epigenetic mechanism consisting of the recruitment of the REST (repressor element 1-silencing transcription factor)/Sp3 transcription factor/histone deacetylase (HDAC)1/HDAC2 complex that deacetylates histones on *brain* promoter sequence in rat neurons.[3] Among the epigenetic modifications, although DNA methylation has been widely studied in brain ischemia,[4] its role in modulating *Ncx1* expression in the

Correspondence to: Luigi Formisano, MD, PhD, Division of Pharmacology, Department of Neurosciences, Reproductive and Odontostomatological Sciences School of Medicine, University of Naples Federico II, Via Sergio Pansini 5, 80131 Naples, Italy. Email: luigi.formisano@unina.it

This article was sent to Neel S. Singhal, MD, PhD, Associate Editor, for review by expert referees, editorial decision, and final disposition.

Supplemental Material is available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.123.030460

For Sources of Funding and Disclosures, see page 16.

JAHA is available at: www.ahajournals.org/journal/jaha

RESEARCH PERSPECTIVE

What Is New?

- The transcriptional repressor REST (repressor element 1-silencing transcription factor) downregulates its target gene *Ncx1* through the binding of a new repressor element 1 motif on Ncx1 heart promoter in the brain.
- REST, by recruiting DNA-methyltransferase-1 and MeCP2 (methyl-CpG binding protein 2) on *Ncx1* heart promoter sequence, hypermethylates *Ncx1* gene, reducing its expression in the cerebral cortex of ischemic mice.

What Question Should Be Addressed Next?

- Future studies could focus on (1) identifying other potential REST/DNA-methyltransferase-1/MeCP2 gene targets involved in neuronal damage after stroke; (2) studying whether reverting DNA methylation, by activating DNA hydroxymethylation, could be a new pharmacological strategy for stroke intervention.

Nonstandard Abbreviations and Acronyms

Br	Ncx1 brain promoter
Ht	Ncx1 heart promoter
Ht-RE1	RE1-site on Ncx1 heart promoter sequence
OGD	oxygen and glucose deprivation

brain has never been investigated. DNA methylation mediates gene repression via the epigenetic writers DNA methyltransferases (DNMTs), which add a methyl group to position 5′ of the cytosine of DNA (5-meC),[5] and via the epigenetic readers, such as MBDs (methyl CpG binding proteins), which specifically recognize the 5-meC.[6] Among the MBDs, the MeCP2 (methyl CpG binding protein 2) protein is of particular interest, because its mutations cause an X-linked neurodevelopmental disorder named Rett syndrome.[7] MeCP2 activates or represses gene transcription[8] depending on its ability to recruit transcriptional activators, such as CREB1 (cyclic AMP-responsive element-binding protein 1),[9] or transcriptional repressors, including REST.[10] Interestingly, REST can recruit DNMT1 and MeCP2 to downregulate some neuronal genes, including BDNF (brain-derived neurotrophic factor) and synapsin 1.[11,12] MeCP2 is predominantly expressed in mature postmitotic neurons,[13] where its expression levels are finely controlled; indeed its doubling expression is responsible for the MeCP2 duplication syndrome, a neurological disease characterized by autism, progressive spasticity, and epilepsy.[14] Accordingly, the overexpression of 1 of the 2 MeCP2 splicing isoforms causes neurotoxicity.[15] Importantly, MeCP2 perturbations and increased DNA methylation levels have been described in in vitro and in vivo models of stroke.[16] Interestingly, drugs inhibiting DNA methylation, such as 5-azacytidine (5-Aza) and zebularine, exert a neuroprotective effect in brain ischemia.[17]

Transcription of *Ncx1* gene is driven by 3 different promoters that have been identified in human, mouse, rat, and cat genomes and were named "*heart*" (*Ht*), "*brain*" (*Br*), and "*kidney*" (*Kc*) according to the tissue specificity of their activation.[18] On the other hand, single promoters or different combinations of these sequences were found driving the expression of *Ncx1* in different anatomical regions,[18] showing that *Ht*, *Br*, and *Kc* also regulate the expression of the exchanger in other tissues. Interestingly, here we found a putative binding site for REST (RE1) in the *Ht* promoter sequence. In light of these new data, we investigated the role of this RE1 site in the regulation of *Ncx1* gene by DNA methylation in in vitro and in vivo models of stroke.

METHODS

Availability of Data and Material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Reagents

All common reagents were purchased from Sigma (Milan, Italy). Enzymes, luciferase reporter kits, and luciferase vectors were purchased from Promega (Milan, Italy).

The following small interfering RNAs (siRNAs) have been used: mouse DNMT1 (sc 35 203, Santa Cruz Biotechnology); mouse MeCP2, HDAC1, HDAC2, NCX1 (SI00195594, SI02732982, SI00173551, SI01424920 QIAGEN); mouse REST (EMU029201, SIGMA); and human DNMT1, REST (SI02663409, SI02929693, QIAGEN); and human MeCP2 (EMU030271, SIGMA). The siCTL (1 027 280, QIAGEN) was used as scramble. 5-Aza from Sigma (A2385) and MS275[3,19] were dissolved in dimethyl sulfoxide and diluted before application to a final dimethyl sulfoxide concentration lower than 0.1%. Plasmids were purchased from Addgene: DNMT1 (cod: 36939),[20] MeCP2 (cod: 110186),[21] and REST.[19] The plasmid pcDNA3.1 was used as empty vector.

Cell Cultures

Human SH-SY5Y cells and U87 cells were purchased from IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy, and grown in DMEM (Invitrogen, Milan, Italy) supplemented with 10% fetal bovine serum in a humidified incubator with 5% CO_2 at 37 °C and usually passaged every 3 to 4 days.[22]

Primary cortical neurons were prepared from 17-day-old Wistar mouse embryos (Charles River, Calco, Italy) and used after 7 to 9 days. Cytosine arabinoside (10 μmol/L) was added on the second day to reduce glial contamination. The experiments on primary cortical neurons were performed according to the experimental protocols approved by the ethics committee of "Federico II" University of Naples and Ministry of Health, Italy (Protocol number 981/217-PR). Briefly, dissection and dissociation were performed in Ca^{2+}/Mg^{2+} free PBS containing glucose (30 mmol/L). Tissues were incubated with papain for 10 minutes at 37 °C and dissociated by trituration in Earl's Balanced Salt Solution containing DNAse (0.16 U/mL) and ovomucoid (10 mg/mL). Neurons, plated in plastic Petri dishes (Falcon™ Becton-Dickinson, Buccinasco, Italy) precoated with poly-D-lysine (20 μg/mL), were grown in MEM/F12 containing glucose, deactivated fetal bovine serum (5%), horse serum (5%), glutamine (2 mmol/L), penicillin (50 Units/mL), and streptomycin (50 μg/mL) (Invitrogen, Milan, Italy).

SH-SY5Y cells, U87 cells, and primary cortical neurons were plated at the following density: for MTT assay in 24-well plates at a density of 1×10^6; for luciferase assay they were plated in 12-well plates at a density of 2×10^6; for quantitative real-time PCR (qRT-PCR) they were plated in 60-mm well plates at a density of 5×10^6 cells/well; for Western blot and chromatin immunoprecipitation (ChIP) analyses they were plated in 100-mm well plates at a density of 15×10^6cells/well.

Transfection With Plasmids or Small Interfering RNA and Luciferase Reporter Assay

SH-SY5Y, U87 cells and primary cortical neurons were transfected with 50 nmol/L of siDNMT1, siMeCP2, or siREST. To overexpress DNMT1, MeCP2, or REST, cells were transfected with 15 μg of each construct in 100 mm plates, 5 μg of each construct in 60 mm plates. Each transfection was performed with Lipofectamine 2000 (11 668 019, Invitrogen, Milan, Italy) in Opti-MEM, as recommended by the producer. Cortical neurons were transfected at DIV 5, whereas SH-SY5Y and U87 cells were transfected 24 hours after plating. All experimental groups involving siRNAs or plasmids transfection are compared with cells transfected with a scramble siRNA (siCTL) or an empty vector (EV), respectively.

For luciferase assay experiments, cells were transfected in 12-well plates. Cells were transfected with a reporter plasmid (800 ng each), among those listed: (1) the pGL3basic construct, (2) the Ncx1 *Br* promoter construct inserted in pGL3basic (*Br-Ncx1* promoter), (3) the pGL3Ht (*Ht-Ncx1* promoter), and (4) the Ncx1 *Ht* promoter construct with a mutation in the RE1 site inserted in pGL3basic; a thymidine kinase promoter-Renilla luciferase reporter plasmid vector (200 ng) expressing the Renilla luciferase gene and used as internal control; DNMT1 vector, MeCP2 vector, or REST vector, as overexpressing plasmids, and pcDNA3.1 as empty vector (1 μg each). The treatments with 5-Aza (5 μmol/L)[23,24] were performed 24 hours after transfection for 24 hours, and treatment with MS275 (1 μmol/L) was as previously reported.[19] All experimental groups involving drug transfection are compared with cells treated with a vehicle (0.1% dimethyl sulfoxide). The promoter activity was measured 48 hours after the transfection by using the Dual-Luciferase Reporter Assay System kit (E1910) (Promega, Milan, Italy), as suggested by the producer.

Cloning and Mutagenesis

PCR amplification was performed using PrimeSTAR GLX DNA polymerase (Takara, Japan) on human genomic DNA, priming a region of 473bp from 40 512 263 to 40 512 735 of chromosome 2 (GRCh38/hg38). The primers used for the amplification included XhoI and EcoRI restriction sites, absent in the target gene. The endonucleases sites were incorporated in the forward and reverse primers when a target region was generated by PCR (Fw primer: 5′-ATAGAGAG TAGAAATGCCAATCTC-3′; Rv primer: 5′-GGCCCTTA AAGGACATCAAGGG-3′; endonucleases binding sites are underlined). The PCR product was purified using StrataPrep DNA gel extraction kits (Agilent, Milan, Italy) and cloned into multiple cloning sites of pGL3-basic luciferase expression vector (Promega, Milan, Italy). The fidelity of the constructs was confirmed by DNA sequencing. Site-directed mutations were carried out in the REST binding sequence using the quick change site-directed mutagenesis kit (Agilent) using the forward primer: 5′-CTGTTGGGAACAGATCC ATGGAAGCGAAAGCCGAAAG-3′ and the reverse primer 5′-CTTTCGGCTTTCGCTTCCATGGATCTGTT CCCAACAG-3′ (mutated bases are underlined), according to the manufacturer's instructions, as previously reported.[3] Finally, mutations were confirmed by DNA sequencing.

Combined Oxygen and Glucose Deprivation and Reoxygenation

Oxygen and glucose deprivation (OGD) was performed in mouse cortical neurons in an OGD-dedicated

medium previously saturated with 95% N_2 and 5% CO_2 and containing NaCl 116 mM, KCl 5.4 mmol/L, $MgSO_4$ 0.8 mmol/L, $NaHCO_3$ 26.2 mmol/L, NaH_2PO_4 1 mmol/L, $CaCl_2$ 1.8 mmol/L, glycine 0.01 mmol/L, and 0.001% w/v phenol red. Specifically, cells were placed in a hypoxic chamber for 4 hours, in the following condition: temperature 37 °C, atmosphere 5% CO2, and 95% N2. To block OGD, cells were removed from the hypoxic chamber and placed in a normal medium for 24 hours of reoxygenation.[25] Cells maintained in growth medium under normoxic conditions were used as controls (CTL).

In Vivo Studies

Experimental Groups

One hundred and eight C57 male 8-week-old mice (Charles River) weighing 22 to 24 g were housed under diurnal lighting conditions (12 hour darkness/light) and were maintained in individually ventilated cages. To ensure an optimal acclimatization period, the animals were operated 96 hours after arrival in the animal facility.

Animals not showing a reduction in cerebral blood flow of at least 70% and those dying after ischemia induction, which represents 20% of total mice used, were excluded from the study. Animals were randomly allocated in the different experimental groups, the treatment with each siRNA was blindly performed, and the analysis was performed by a researcher blinded to the applied treatment. Sample size (n=3-5/group) for stroke experiment measurements was calculated a priori by using G-power software and by predicting a conventional effect size of 0.9 and an alpha error of 0.1 and a statistical power of 95%. For all experiments, freshly isolated brains were covered and embedded in saline solution on dry ice. Experiments were performed according to the international guidelines for animal research and approved by the Animal Care Committee of Federico II University of Naples, Italy and Ministry of Health, Italy (Protocol number 981/217-PR). We used Animal Research: Reporting of In Vivo Experiments guidelines for this study.[26]

Focal Transient Brain Ischemia

Transient focal ischemia was induced, as previously described[22] by occlusion of the middle cerebral artery (MCA) in male mice anesthetized using 1.5% sevoflurane and 98.5% O_2. Briefly, a 5-O surgical monofilament nylon suture (Doccol, CA, USA) was inserted through the external carotid artery stump and advanced into the left internal carotid artery until it blocked the origin of the MCA.[27] Sham-operated animals (Sham) were subjected to the same surgical procedure without insertion of the filament and used as control group for the in vivo experiments. Achievement of ischemia was confirmed by monitoring regional cerebral blood flow in the area of the right MCA. Cerebral blood flow was monitored through a disposable microtip fiber optic probe (diameter 0.5 mm) connected through a master probe to a laser Doppler computerized main unit (PF5001; Perimed, Sweden) and analyzed using PSW Perisoft 2.5.[28] Rectal temperature was maintained at 37±0.5 °C with a thermostatically controlled heating pad and lamp. All surgical procedures were performed under an operating stereomicroscope. After 60 minutes MCA occlusion, mice were reanesthetized and the filament was withdrawn in order to restore blood flow. After the operation, mice recovered in their individual cages. In order to reduce the suffering of the animals, a constant analgesic-anesthetic treatment has been used during and after surgical procedures. Upon awakening, the animals will be provided with appropriate analgesic treatment and continuous monitoring by the veterinary doctor. Mice were euthanized at 6, 12, and 24 hours after transient MCA occlusion (tMCAO), and the temporoparietal cortices were harvested for PCR, immunoprecipitations, ChIP, and Western blot analysis.

siRNA Administration

For siRNAs administration, mice were positioned on a stereotaxic frame, and siRNAs were injected through a 27-g stainless steel guide cannula implanted into the right lateral ventricle using the stereotaxic coordinates from the bregma: +0.6 mm caudal, –1.6 mm lateral, and 2.3 mm below the dura. siRNAs (Cf 10 μmol/L, 1 μL) administration was performed 24 hours before and after ischemia induction as previously reported.[25] All experimental groups involving siRNA administration are compared with animals treated with a scramble siRNA (siCTL).

Quantitative Real-Time PCR Analysis

First-strand cDNA and qRT-PCR were carried out as previously described. In brief, qRT-PCR was carried out in a 7500-fast RT-PCR system (Applied Biosystems, Monza, Italy) with SYBR Green Master Mix (cod. 4 385 610; Applied Biosystems, Monza, Italy). Triplicate samples were amplified simultaneously in one assay as follows: heating 2 minutes at 50 °C, denaturation 10 minutes at 95 °C, amplification and quantification 40 cycles of 30 sec at 60 °C, with a single fluorescence measurement. PCR data were collected by using ABI Prism 7000 SDS software (Applied Biosystems). The oligonucleotide sequences for *Ncx1* were designed on the Exon 2 (m): (FW 5′-CAATGGAGAGACCACCA AGAC-3′ and RV 5′-GTCTCCTGCAGTGAAGTTATGG –3′); for DNMT1 (m): (FW 5′- ACCTAGACGACCCTAAC CTGAAGTAC-3′ and RV 5′-GTGGAGTCG TAGATGGA CAGTTTCT-3′); for MeCP2 (m): (FW 5′-AGACAACTCTG

TTCAGTGCTATCACA –3′ and RV 5′-GCA ACG GAAAG CTGAATAAAGC-3′); for REST (m): (FW 5′-TCCGCATG TGTCGCGTTA-3′ and RV 5′-GTTCAGCACGTGCGA ACTCA-3′); for DNMT1 (h): (FW 5′-AACGGCATCCT GTACCGAGTT-3′ and RV 5′-CCTTCCGTGGGC GTT C-3′); for MeCP2 (h): FW 5′-GCGAGAGCAGAAACCAC CTAA-3′ and RV 5′-TGCCAGTTCCTGGAG CTTTG-3′; for *Ncx1* (h) and for REST (h) the oligonucleotide sequences were already published.[19] Data were normalized with respect to GAPDH for mouse genes, and to Ribosomal Protein L19 for human genes.[19] Changes in gene expression between groups were represented as the mean of the relative quantification values, which was calculated as the difference in threshold cycle (ΔCt) between the target gene and the reference gene ($2^{-\Delta\Delta CT}$= relative quantification).

Western Blotting and Immunoprecipitation

Experiments were performed as previously reported.[22] Proteins were separated for NCX1, DNMT1, MeCP2, and REST Western blotting on 8% SDS polyacrylamide gels. All were transferred onto Hybond ECL nitrocellulose membranes (Amersham, Milan, Italy). Membranes were blocked with 5% nonfat dry milk in 0.1% Tween 20 (Sigma, Milan, Italy) for 2 hours at room temperature, and then they were incubated overnight at 4°C in the blocking buffer with the following antibodies: anti-NCX1 (Rabbit pAB cod: p11-13 SWANT, dilution 1:1000), anti-MeCP2 (Rabbit mAb, cod: 3456 Cell Signaling, dilution 1:1000), anti-DNMT1 (Mouse mAb, cod: sc-271 729 Santa Cruz Biotechnology, dilution 1:500), anti-REST (Rabbit pAb, cod: 07–579 Sigma-Aldrich, dilution 1:1000), and anti-β-actin (cod: A3854 Sigma Aldrich, dilution 1:10 000). After the incubation with primary antibodies, membranes were washed with 0.1% Tween 20, followed by incubation with secondary antibodies for 1 hour at room temperature.

For Immunoprecipitation experiments, tissue lysates from perischemic cortex of mouse treated with siCTL or siREST were performed by using immunoprecipitation lysis buffer (50 mmol/L Tris–HCL, pH 7.5, 1, Triton 1%, β-glicerol 10 mmol/L, NaF 100 mmol/L, Na3VO4 100 mmol/L, 150 mmol/L NaCl, and 1 mmol/L EDTA), with the addition of protease inhibitor cocktail (cod: P-8140, Sigma-Aldrich). Aliquots of 1000 μg were immunoprecipitated overnight at 4 °C using 3 μg of rabbit polyclonal anti-REST or normal rabbit IgG. Next, 20 μL of protein A/G PLUS-agarose beads (cod: sc-2003, Santa Cruz Biotechnology) was added to each sample to precipitate the proteins bound to the antibodies. After a brief centrifugation, the pellets were washed twice and resuspended in 20 μL of loading buffer, boiled for 10 minutes, and centrifuged. The supernatants were then subjected to Western blot analysis as described previously. Membranes were incubated overnight at 4 °C with the antibodies anti-DNMT1 and anti-MeCP2. Immunoreaction and band density quantification were evaluated as previously reported.[29]

Chromatin Immunoprecipitation

Brain tissues were cross-linked and chromatin was immunoprecipitated as previously reported.[22] Thirty microliters of the preimmunoprecipitated lysate was saved as input for normalization. Chromatin lysates were incubated overnight with 3 μg of antibody for DNMT1 (Mouse mAb, cod: sc-271 729 Santa Cruz Biotechnology, dilution 1:500), MeCP2 (Rabbit mAb, cod: 3456 Cell Signaling, dilution 1:1000), REST, HDAC1, and HDAC2.[3] Normal rabbit IgG was used as negative control. After immunoprecipitation, 40 μL of salmon sperm DNA/protein A-agarose beads (16–157, Millipore, Milan, Italy) was added to the lysates in order to precipitate the proteins bound to DNA. After rotating for 2 hours at 4 °C on a spinning wheel, the beads were washed and precipitates as previously reported.[22] The obtained DNA fragments were first purified by phenol–chloroform extraction, ethanol precipitation, and then dissolved in sterile water. DNA was analyzed by qRT-PCR using Fast SYBR Green Master Mix (cod: 4 385 610, Applied Biosystems). The oligonucleotides used for the amplification of the immunoprecipitated DNA of mouse *Br* were the following: FW 5′-CGCCG GCTTTGTGTTACC-3′ and RV 5′-CGCTCACTCCC TTTTGAGACA-3′, whereas for mouse *Ht* were: FW 5′-TTCTCGACAGCTTGGCGTTA-3′ and RV 5′-CGGCT TTCGCTTCCATACA-3. In particular, DNA sequences/samples were heated for 2 minutes at 50 °C and denatured for 10 minutes at 95 °C. Amplification and quantification, achieved after 40 cycles at 95 °C for 30 seconds, were followed by single fluorescence measurements for 30 seconds at 60 °C. Binding activity was normalized for the total input of chromatin. The Sham group immunoprecipitated with IgG was defined as 1.0 and was used to compare the other experimental groups. Samples were amplified simultaneously in duplicate in each assay run, and the experiments were repeated at least 3 times.

Transient-Transfection ChIP Assay

ChIP from SH-SY5Y cells was performed as previously described.[3] Cells were harvested by cross-linking with 1% formaldehyde, and ChIP was performed with antibody for REST and IgG as negative control at 48 hours after transfection (data not shown). The input and the precipitated DNA was amplified using the *Ncx1-Br* forward primer: 5′-GAAAGGCACAGATA AGCTG-3′ and a LucN rev primer 5′-CCTTATGCAGT TGCTCTCC–3′.

Bisulfite Sequencing

Genomic DNA was isolated from cortices of tMCAO and sham-operated mice using Wizard Genomic DNA Purification Kit (Promega), according to the manufacturer's protocol. Extracted DNA was subjected to 2 rounds of proteinase K digestion in 10 mM Tris HCl pH 7.8, 5mM EDTA, 0.5% SDS (vol/vol), and 5 μg proteinase K (Roche) for 2 hour at 55 °C; 0.5 μg of genomic DNA was subjected to bisulfite modification with the Epitect Bisulfite kit (Qiagen) and eluted in 40 μL. PCR was performed to amplify target regions from bisulfite-modified genomic DNA. GpG group 1 region was amplified using NCX1_CpG_group1_R1 (5′-CTCCC CTACAAACATCAAAAACTCTAC-3′) and NCX1_CpG_group_F2 (5′-GAATTAAGTAAGTTTATATTTTG TAAATTTTG-3′). "CpG island 2" region was amplified using NCX1_CpGi_island2_F2 (5′-GGAAATTTAGTTAT TGGTAGAGTAGAGGAG-3′) and NCX1_CpGi_island2_R1 (5′-CCAAAACCAACCCTTTAACTTAAAC-3′). For the amplification of both genomic regions, the thermal cycling conditions were 95 °C for 1 minute, followed by 95 °C for 45 seconds, 55 °C for 45 seconds and 72 °C for 45 seconds, for 40 or 35 cycles (for "CpG island 1" or "GpG island 2," respectively), with an additional step of 5 minutes at 72 °C. Reactions were performed with 1 μL of bisulfite modified genomic DNA in the presence of 0.25 μmol/L of each primer, 0.5 units of MyTaq horse serum DNA polymerase (Bioline) and 1X MyTaq reaction buffer. PCR products were recovered from agarose gels using QIAquick Gel Extraction Kit (Qiagen), following the manufacturer's instructions. Purified PCR fragments were cloned in the pCR2.1 cloning vector using TA Cloning Kit (Invitrogen) and sequenced by using the dye terminator method (Eurofins Genomics). The sequencing of 10 clones for each sample was performed using M13 reverse primers.

Confocal Double Immunofluorescence

Confocal immunofluorescence procedures were performed as previously described.[25] Briefly, mice were euthanized 24 hours after sham surgery or tMCAO. Mice were anesthetized intraperitoneally with chloral hydrate (300 mg/kg) and perfused transcardially with 4% w/v paraformaldehyde in phosphate buffer. The brains were sectioned coronally (50 μm), and after blockade sections were incubated with the following primary antibodies: anti-DNMT1 (Mouse mAb, cod: sc-271 729 Santa Cruz Biotechnology, dilution 1:500), anti-MeCP2 (Rabbit mAb, cod: 3456 Cell Signaling, dilution 1:1000), anti-NeuN (Rabbit pAb, cod: ABN78, Merck Millipore, dilution 1:1000) and Hoechst (cod: 33342, Thermo Fisher, dilution 1:1000). Subsequently, sections were incubated in a mixture of the fluorescent-labeled secondary antibodies (Alexa 488/Alexa 594-conjugated antimouse/antirabbit IgG). Images were observed with a Zeiss LSM510 META/laser scanning confocal microscope. Single images were acquired with an optical thickness of 0.7 m and a resolution of 1024×1024.

Evaluation of the Infarct Volume

For the evaluation of the ischemic volume, animals were killed with sevoflurane overdose 24 hours after ischemia. Brains were quickly removed, sectioned coronally at 1 mm intervals, and stained by immersion in the vital dye (2% in PBS) 2,3,5-triphenyltetrazolium hydrochloride. The infarct volume was calculated by summing the infarction areas of all sections and by multiplying the total by slice thickness. To avoid the edema affecting the infarct volume value, the infarct volume was expressed as percentage of the ischemic damage by dividing the total infarct volume by the total ipsilateral hemispheric volume.

Statistical Analysis

Data were analyzed by GraphPad Prism 5 software (Graph PadSoftware, Inc). All bars in the figures represent the mean±SD. For statistical analysis, all data were log-transformed to obtain normal distribution of the data and then analyzed with the Student's *t* test and 1-way ANOVA. Specifically, statistically significant differences between 2 experimental groups were evaluated by Student's *t* test. Statistically significant differences between more than 2 experimental groups were evaluated by 1-way ANOVA, followed by Tukey multiple comparison test, whereas a 1-way ANOVA followed by Newman–Keuls test was used to analyze the significance of the infarct volume measurements. Statistical significance was accepted at the 95% CI ($P<0.05$).

RESULTS

DNMT1, MeCP2, and REST Negatively Modulated *Ncx1* mRNA and Protein Expression in Neuronal SH-SY5Y Cells

To study the effect of DNMT1, MeCP2, and REST complex on the regulation of *Ncx1* transcription, SH-SY5Y cells were transfected with siDNMT1, siMeCP2, and siREST. siDNMT1 and siMeCP2 reduced the mRNA expression of DNMT1 and MeCP2 by 54% and 56%, respectively (Figure 1A and 1B), whereas siREST downregulated *REST* expression by 40%.[30] Interestingly, DNMT1, MeCP2, and REST silencing significantly increased both *Ncx1* mRNA and protein expression, compared with cells transfected with siCTL (Figure 1C). To further confirm the role of DNMT1, MeCP2, and REST in the modulation of *Ncx1* expression, SH-SY5Y cells were transfected with constructs overexpressing DNMT1, MeCP2, or REST. These constructs increased *DNMT1* and MeCP2 mRNA expression by 142% and

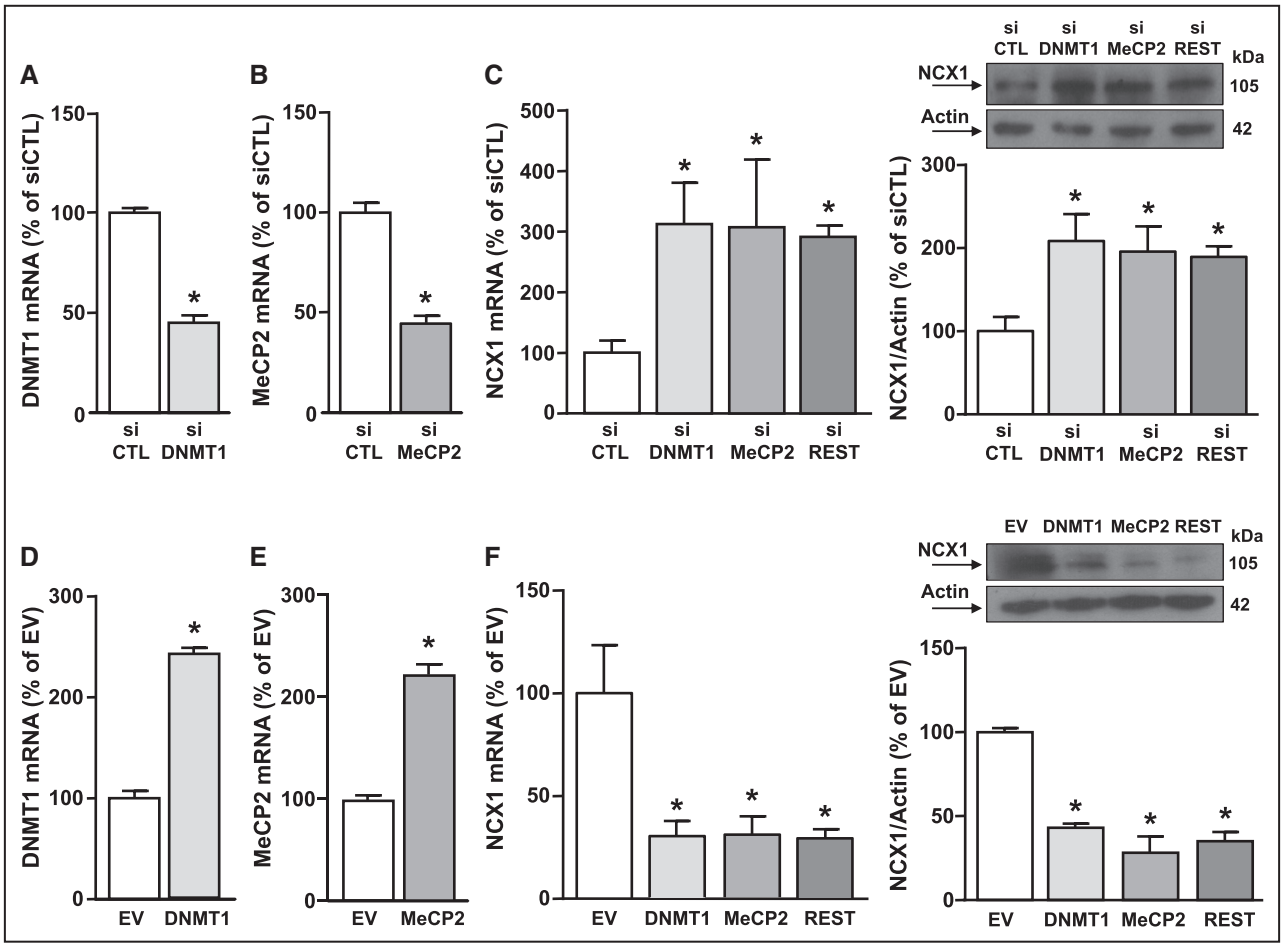


Figure 1. The epigenetic factors DNMT1 and MeCP2 and the transcriptional factor REST are involved in the negative modulation of *Ncx1* mRNA and protein expression.
A and **B**, qRT-PCR for DNMT1 and MECP2 transcripts in SH-SY5Y cells transfected for 48 hours with siDNMT1 or siMeCP2 at a final concentration of 50 nM. An siCTL was used as scrambled. *$P \leq 0.05$ vs siCTL by Student's *t* test (n=3). **C**, *Ncx1* mRNA and protein expression in SH-SY5Y cells transfected for 48 hours with siCTL, siDNMT1, siMeCP2, and siREST. *$P \leq 0.05$ vs siCTL by 1-way ANOVA analysis followed by Tukey's post hoc test (n=3/4). **D** and **E**, qRT-PCR for DNMT1 and MECP2 transcripts in SH-SY5Y transfected for 48 hours with vector overexpressing DNMT1 or MeCP2. pcDNA3.1 vector was used as EV. *$P \leq 0.05$ vs EV by Student's *t* test (n=3). **F**, *Ncx1* mRNA and protein expression in SH-SY5Y cells transfected for 48 hour with EV, DNMT1, MeCP2, or REST plasmids. *$P \leq 0.05$ vs EV by 1-way ANOVA analysis followed by Tukey's post hoc test (n=3/4). CTL indicates control; DNMT1, DNA-methyltransferase-1; EV, empty vector; MeCP2, methyl-CpG binding protein 2; NCX1, sodium/calcium exchanger 1; qRT-PCR, quantitative real time polymerase chain reaction; REST, repressor element 1-silencing transcription factor; si, small interfering; and siCTL, control siRNA.

123%, respectively (Figure 1D and 1E), whereas REST transfection increases the protein expression by 75%, as previously reported.[19] Cells overexpressing DNMT1, MeCP2, and REST showed a significant reduction in *Ncx1* transcript and protein levels, compared with cells transfected with the empty vector (Figure 1F).

DNMT1 and MeCP2 Reduced *Ht* But Not *Br Ncx1* Promoter Activity in Neuronal SH-SY5Y Cells

Ncx1 transcript levels are regulated in the brain through the activation of the *Br* promoter[31] and *Ht* promoter.[32] Intriguingly, human *Ht* showed a sequence identity of 78.07% and 76.87% with mouse and rat ortholog regions, respectively (Figure 2A). Because these promoters are not tissue specific,[18] we investigated which *Ncx1* promoter sequence is regulated by DNMT1, MeCP2, and REST in neuronal cells.

To this aim, we cloned a human genomic region containing *Ncx1-Ht* sequence (–300/+73, ENST00000402441.5, Chr2:40512135-40512422) in the promoter-less firefly luciferase-based reporter vector (pGL3basic), whereas vector containing *Br* sequence was cloned as previously reported.[31] Luciferase experiments showed that siREST increased both *Br* and *Ht* promoter activity, whereas siDNMT1 and siMECP2 increased the activity of *Ht* promoter but had no effect

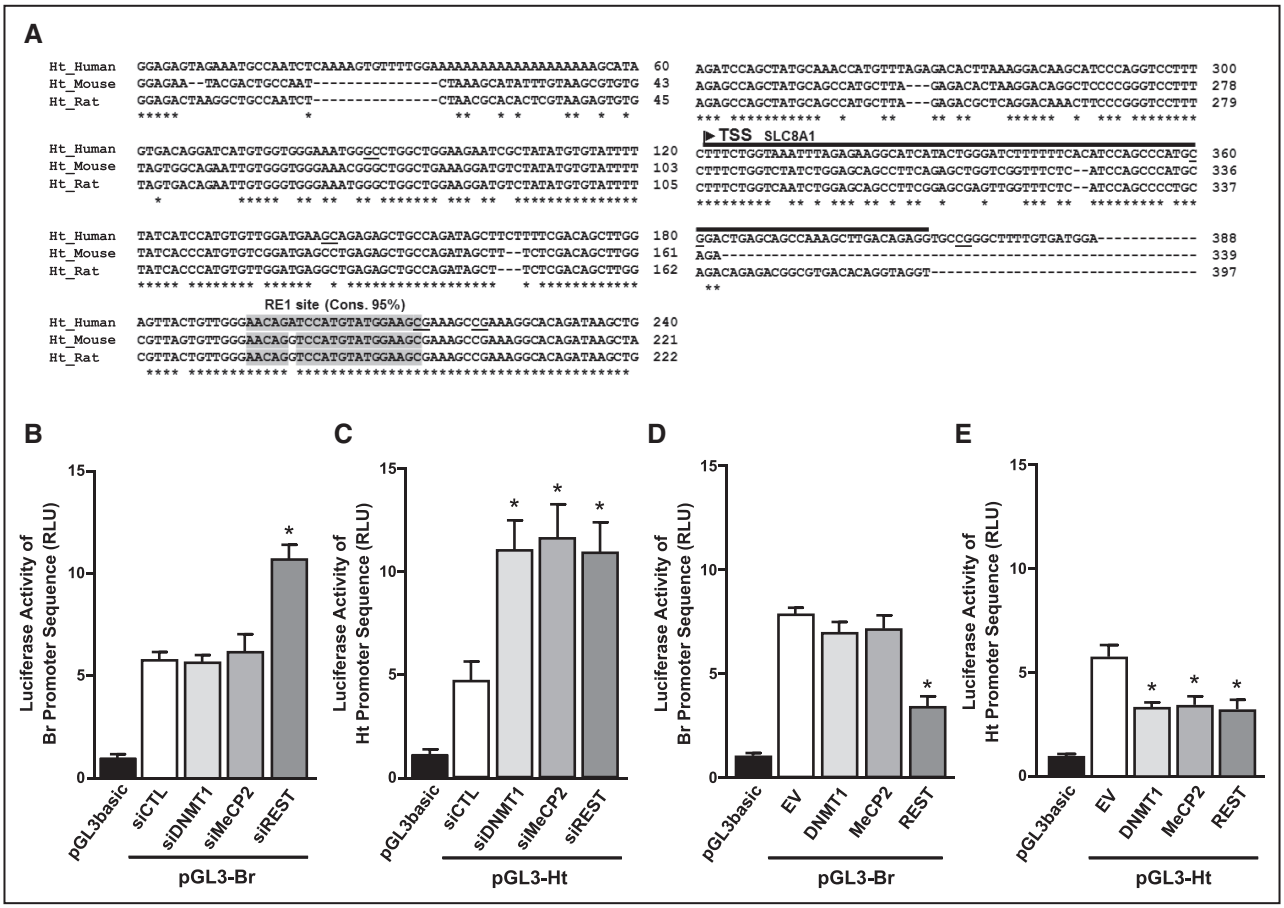


Figure 2. The epigenetic factors DNMT1 and MeCP2 and the transcriptional factor REST are involved in the negative modulation of *Ncx1* promoter activity.

A, Alignment of the human, mouse, and rat *Ht* sequences is shown. Conserved nucleotides are marked with asterisk. The arrow indicates the TSS. **B** through **E**, Luciferase assay in SH-SY5Y cells transfected for 48 hour with pGL3-Br (**B** and **D**) or pGL3-Ht (**C** and **E**) and cotransfected with siCTL, siDNMT1, siMeCP2, or siREST (**B**, **C**), or with EV, DNMT1, MeCP2, or REST (**D** and **E**). **P*≤0.05 vs siCTL or EV by 1-way ANOVA analysis followed by Tukey's post hoc test (n=4). CTL indicates control; DNMT1, DNA-methyltransferase-1; EV, empty vector; *Ht*, heart promoter, MeCP2, methyl-CpG binding protein 2; NCX1, sodium/calcium exchanger 1; pGL3-Br, Ncx1 brain promoter construct inserted in pGL3basic; pGL3-Ht, Ncx1 heart promoter construct inserted in pGL3basic; REST, repressor element 1-silencing transcription factor; si, small interfering; and TSS, transcription start site.

on *Br* promoter activity (Figure 2B and 2C). In addition, the transcriptional activity of Ncx1 *Ht* promoter construct inserted in pGL3basic construct was significantly inhibited by 43%, 41%, and 44% in cells overexpressing DNMT1, MeCP2, and REST, respectively (Figure 2E). Interestingly, overexpression of REST, but not of DNMT1 and MeCP2, reduced *Br* promoter activity by almost 45% (Figure 2D). Furthermore, we evaluated the effect of DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine (5-Aza)[33] to modulate *Ht* and *Br* promoter activity in mouse cortical neurons. Results showed that the demethylating agent 5-Aza reduced DNMT1 protein expression, thus indicating that the drug leads to a lower DNA methylation by depriving DNMT1 activity, as previously reported[33] (Figure S1A).

As shown in Figure S1B, 5-Aza treatment (5 μmol/L) increased *Ht* activity but did not affect Ncx1 *Br* promoter construct inserted in pGL3basic luciferase activity in cortical neurons. Conversely, HDAC class I inhibitor MS275 (5 μmol/L) significantly increased *Br* promoter activity but did not modify Ncx1 *Ht* promotre construct inserted in pGL3basic luciferase activity (Figure S1B). These results indicate that in neurons the histone deacetylation epigenetic mechanism controls *Br* promoter function, whereas *Ht* promoter is regulated by DNA methylation. In order to verify whether the effects of DNMT1/MeCP2/REST complex or 5-Aza on ncx1 *Ht* promoter activity observed in neurons also occurred in glial cells, we used U87 human glial cells. Notably, 5-Aza or transfection with siREST, siDNMT1, or siMeCP2 did not modify the transcriptional activity of *Ht* promoter in human U87 glial cells (Figure S1C), suggesting that this mechanism occurred specifically in neurons.

REST Bound *Ht*-RE1 on *Ncx1 Ht* Promoter and Reduced Its Transcriptional Activity

REST is known to regulate the expression of its target genes by binding a 21 bp canonical RE1 motif.[34] Through the use of Jaspar database[35] we found a RE1 element in human *Ht* sequence (*Ht*-RE1) located on the forward DNA strand from nucleotides –108 to –88 relative to the transcription start site (Figure 3A). Accordingly, mutation of *Ht*-RE1 sequence (Ncx1 *Ht* promoter construct inserted in pGL3basic-RE1mut) prevented the down-regulation of *Ht* promoter activity induced by REST overexpression in SH-SY5Y cells (Figure 3B). Furthermore, REST overexpression increased its binding to *Ht* exogenous promoter sequence in cells transfected with the Ncx1 *Ht* promoter construct inserted in pGL3basic construct. By contrast, REST overexpression failed to increase its binding to the mutated *Ht*-RE1 sequence (Figure 3C). These results indicated that REST requires *Ht*-RE1 to repress *Ncx1* transcription in neuronal cells.

tMCAO Caused an Increase in DNMT1, MeCP2, and REST Expression Levels in the Peri-Infarct Temporoparietal Cortex; Their Binding to Heart Sequence; and Heart Promoter DNA Methylation

REST levels significantly increased at 12 and 24 hours in peri-infarct temporoparietal cortex in mice after stroke,

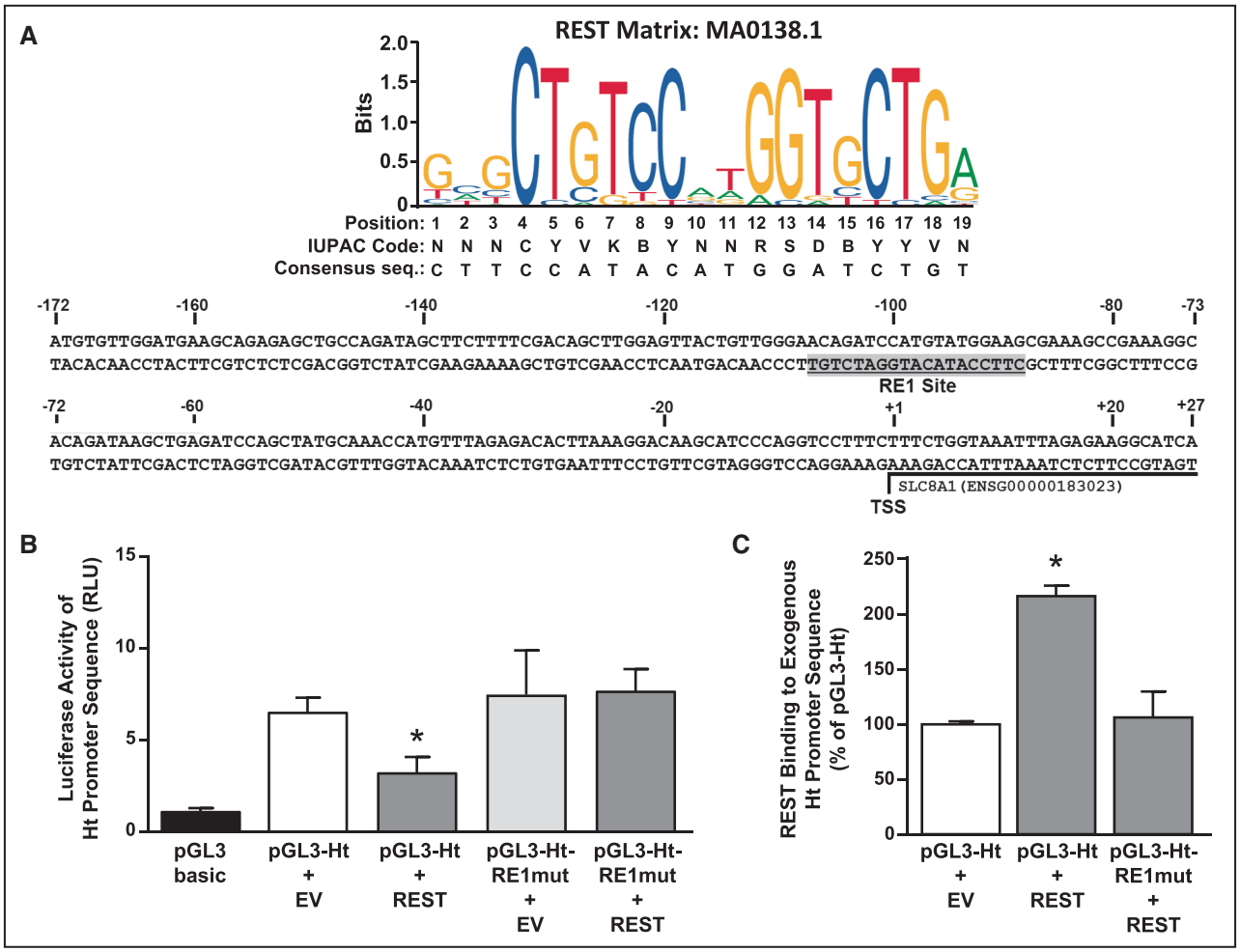


Figure 3. REST represses *Ncx1* transcription through a direct binding of a RE1 site on *Ht* promoter region.
A, Top: JASPAR matrix representation (MA0138.1) of the consensus REST binding site on *Ht* gene (*Ht*-RE1). *Ht*-RE1 sequence is represented in International Union of Pure and Applied Chemistry code. Bottom: Partial human genomic *Ht* sequence containing the predicted REST binding site (underlined). **B**, Luciferase assay in SH-SY5Y cells under the following experimental conditions: (1) pGL3basic, (2) pGL3-Ht+EV, (3) pGL3-Ht+REST, (4) pGL3-Ht-RE1mut+EV, (5) pGL3-Ht-RE1mut+REST. *$P \leq 0.05$ vs pGL3Ht+EV by 1-way ANOVA analysis followed by Tukey's post hoc test (n=4). **C**, ChIP with anti-REST antibody followed by qPCR of the promoter region containing the RE1 site on the *Ht* gene in SH-SY5Y cells transiently transfected with (1) pGL3-Ht+EV, (2) pGL3-Ht+REST, (3) pGL3-Ht+RE1mut+REST. *$P \leq 0.05$ vs pGL3-Ht+EV by 1-way ANOVA analysis followed by Tukey's post hoc test (n=4). ChIP indicates chromatin immunoprecipitation; CTL, control; DNMT1, DNA-methyltransferase-1; EV, empty vector; *Ht*, heart promoter, MeCP2, methyl-CpG binding protein 2; NCX1, sodium/calcium exchanger 1; pGL3-Br, Ncx1 brain promoter construct inserted in pGL3basic; pGL3-Ht, Ncx1 heart promoter construct inserted in pGL3basic; qRT-PCR, quantitative real time polymerase chain reaction; RE1, repressor element 1; and REST, repressor element 1-silencing transcription factor.

whereas DNMT1 and MeCP2 increased at 24 hours, as compared with sham-operated mice (Figure S2A through S2C). Notably, *Ncx1* mRNA and protein were reduced at 12 and 24 hours after stroke (Figure S2D and S2E). Twenty-four hours after tMCAO insult, DNMT1 and MeCP2 expression strongly increased in NeuN positive neuronal nuclei compared with the corresponding ipsilateral cortical regions of sham-operated animals (Figure S3A and S3B). Interestingly, REST and NCX1 were also found to be overexpressed and reduced in the same perischemic brain regions, respectively.[19] ChIP assays showed an increase in REST enrichment on *Br* promoter sequence at 12 hours after tMCAO, whereas it was augmented on *Ht* promoter at 24 hours (Figure 4C). By contrast, DNMT1 and MeCP2 binding occurred on *Ht* promoter only at 24 hours after tMCAO but was unbound on *Br* promoter either at 12 and 24 hours after stroke (Figure 4A and 4B). Notably, HDAC1 and HDAC2 bonded on *Br* promoter only at 12 hours after tMCAO but were absent on *Ht* promoter either at 12 and 24 hours after stroke (Figure 4D and 4E). Furthermore, we investigated whether the silencing of HDAC1 and HDAC2 could influence DNMT1 and MeCP2 binding on *Ncx1-Ht* promoter 24 hours after stroke. As shown in Figures 4A and 4B, HDAC1 and HDAC2 knocking-down does not modify stroke-induced DNMT1 and MeCP2 enrichment on *Ht* promoter.

Because MeCP2 is known to act as a transcriptional repressor through the binding of methylated promoters of a plethora of genes,[8] we investigated the DNA methylation status of *Ncx1* promoters after stroke insult. In particular, we examined 2 regions enriched in CpG sites, corresponding to *Ht* and *Br*

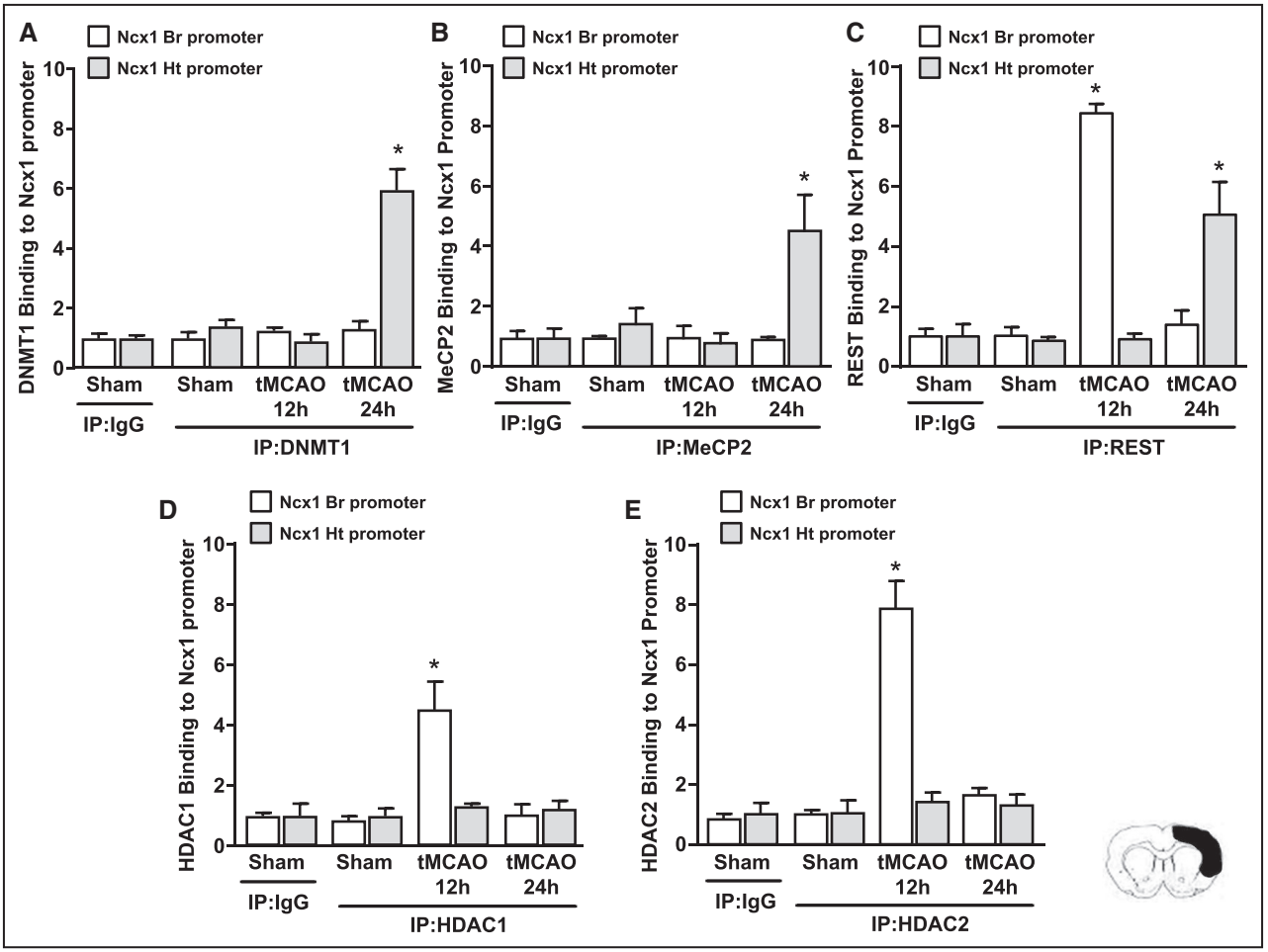


Figure 4. DNMT1 and MeCP2 bind *Ht*, but not *Br* promoter sequence, in the temporoparietal cortex 24 hours after tMCAO. Chromatin immunoprecipitation with anti-DNMT1 (**A**), anti-MeCP2 (**B**) anti-REST (**C**), anti-HDAC1 (**D**), anti-HDAC2 (**E**), antibodies followed by qPCR of *Ncx1* brain promoter (*Br*) (white columns) and *Ncx1* heart promoter (*Ht*) (gray columns) in peri-ischemic cortex of mice euthanized 12 or 24 hours after tMCAO. IgG was used as negative control. *$P \leq 0.05$ vs Sham immunoprecipitated with IgG antibody by 1-way ANOVA analysis followed by Tukey's post hoc test (n=3). DNMT1 indicates DNA-methyltransferase-1; HDAC, histone deacetylase; IP, immunoprecipitation; MeCP2, methyl-CpG binding protein 2; NCX1, sodium/calcium exchanger 1; qRT-PCR, quantitative real time polymerase chain reaction; REST, repressor element 1-silencing transcription factor; and tMCAO, transient middle cerebral artery occlusion.

regions, located upstream to the exon 2, named "CpG group 1" and "CpG island 2," respectively, in the cortex of tMCAO and sham-operated mice. CpG group 1 region, containing *Ht* promoter, is located on chromosome 17 (chromosome 17; position 81 738 227-81 738 737; mouse mm10) and includes 16 CpG sites (Figure 5A and 5B). In this region we found a significant hypermethylation (52.81%) in the peri-infarct temporoparietal cortex at 24 hour after tMCAO, as compared with sham-operated mice (28.13%) (Figure 5B). These results were consistent with the increased binding of both MeCP2 and DNMT1 in this promoter region upon tMCAO insult. CpG island 2 region was located downstream CpG group 1 region (chromosome 17; position 81 669 893-81 670 493; mouse mm10) and contains *Br* promoter sequence and a group of 68 CpG sites (Figure 5A and 5C). However, these CpG sites, in contrast with CpG sites of CpG group 1, appeared almost completely unmethylated in both ischemic (1.18%) and not ischemic (1.56%) cortices (Figure 5C). These results were in accordance with the lack of MeCP2 and DNMT1 binding to this *Br* promoter region we observed (Figure 4A and 4B).

REST Knockdown Reduced MeCP2 and DNMT1 Binding on *Ncx1 Ht* Promoter in the Peri-Infarct Temporoparietal Cortex at 24 Hours After tMCAO

Coimmunoprecipitation experiments confirmed that REST bound to the 2 epigenetic factors DNMT1 and MeCP2 in the temporoparietal cortex of ischemic mice (Figure S5A). The interaction between REST, MeCP2, and DNMT1 was reduced by intracerebroventricular injections of siREST. Interestingly, ChIP analysis revealed that REST silencing significantly counteracted the binding of DNMT1 (Figure S5B), MeCP2 (Figure S5C), and REST (Figure S5D) on *Ht* promoter sequence in cerebral cortex 24 hours after tMCAO, thus suggesting that these 3 factors form a complex. Furthermore, siREST also prevented the reduction in RNA-Pol II binding on *Ht* promoter sequence 24 hours after tMCAO (Figure S5E).

Silencing of DNMT1, MeCP2, and REST Prevented *Ncx1* Downregulation Induced by In Vitro and In Vivo Models of Stroke and Reduced Brain Damage

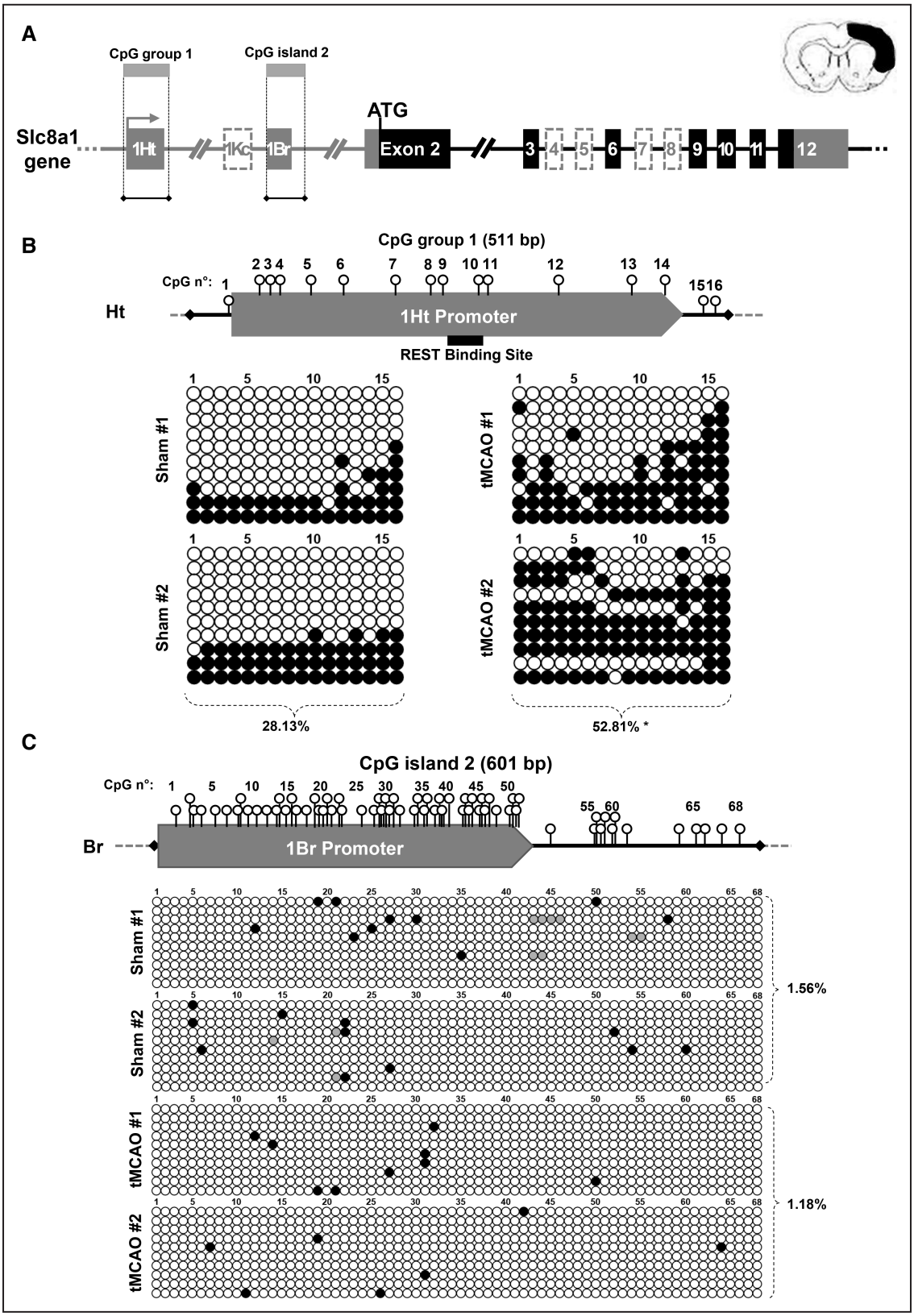
The silencing of DNMT1, MeCP2, or REST obtained by intracerebroventricular injection of their respective siRNAs reduced the expression of these genes by 52%, 72%, and 53%, respectively (Figure S6A through S6C). Interestingly, silencing of REST, DNMT1, or MeCP2 prevented tMCAO-induced reduction of both *Ncx1* mRNA and protein levels (Figure 6A and 6B). Consistently, we found that either siDNMT1, siMeCP2, or siREST treatment were able to reduce the infarct volume that occurred after stroke (Figure 6C). Furthermore, to confirm the correlation between DNMT1/MeCP2/REST complex formation and *Ncx1* downregulation after stroke we used primary mouse cortical neurons subjected to 3 hours of oxygen and glucose deprivation followed by 12 and 24 hours of reoxygenation (OGD/reoxygenation). In this in vitro model mimicking brain ischemia, we observed that REST, DNMT1, and MeCP2 expression were increased after 24-hour OGD/reoxygenation, whereas NCX1 protein expression was decreased at 12 and 24 hours (Figure S7A through S7D). Silencing of DNMT1, MeCP2, and REST, and the DNA methylation inhibitor 5-Aza significantly prevented OGD/reoxygenation-induced *Ncx1* downregulation (Figure S7E and S7F) and consequently the neuronal death (Figure S7G). Interestingly, silencing of *Ncx1* counteracted the neuroprotective effect exerted by the silencing of either siREST, siDNMT1, siMeCP2, or 5-Aza treatment after 24 hour OGD/reoxygenation (Figure S7G).

DISCUSSION

This paper demonstrates for the first time that the transcriptional repressor REST downregulates its target gene *Ncx1*[3] through the binding of a new RE1 motif on *Ncx1 Ht* promoter in ischemic brain. In addition, to our knowledge, we also identified the first gene regulated by 2 RE1 binding sites located on 2 different promoter sequences.

REST downregulated *Ncx1* in stroke by forming a complex with the epigenetic writer DNMT1 and the epigenetic reader MeCP2. The formation of DNMT1/MeCP2/REST complex decreased *Ncx1* mRNA and protein levels in the peri-ischemic temporoparietal cortex through the methylation of the *Ht* DNA sequence. Furthermore, the inhibition of the formation of DNMT1/MeCP2/REST complex showed an amelioration of brain damage by preventing *Ncx1* transcriptional downregulation.

It has been found that *Ht* promoter directs cardiac-specific expression of the exchanger in heart development.[36] However, Scheller and colleagues demonstrated that single promoters or different combinations of these sequences are found to drive the expression of *NCX1* in different anatomical regions, including the brain.[18] In accordance with these papers, our results showed that *Ht* promoter sequence exerts a significant luciferase activity in neuronal cells. More important, we found that the epigenetic factors REST, DNMT1, and MeCP2 were able to downregulate *Ncx1* expression after stroke through the binding of a new identified RE1 motif in *Ht* region on *Ncx1*



promoter. Moreover, disruption of *Ht*-RE1 sequence, by site-directed mutagenesis, prevented both the binding of REST on *Ht* and the downregulation of *Ncx1* upon REST overexpression. Notably, *Ht*-RE1 showed a sequence identity of 95.73% among human, rat, and mouse ortholog regions (Figure 2A). This highly

Figure 5. CpG group 1 containing *Ht* promoter is hypermethylated in peri-ischemic cortex of mice after tMCAO, whereas CpG island 2 remains hypomethylated.

A, Schematic representation of the 5′-region of murine Slc8a1 gene encoding for *Ncx1*. Exons are represented as boxes, introns as thin connecting lines. Black filled boxes correspond to genomic regions encoding for mature protein, whereas gray regions correspond to exons transcribing for untranslated regions. Open boxes represent regions frequently excluded from mature *Ncx1* mRNA by alternative splicing in neurons. Heart, kidney, and brain promoters are represented as 1Ht, 1Kc, and 1Br boxes, respectively. CpG island 1 and 2 are represented above Ht and Br promoter regions. (**B** and **C**) top: schematic representation of CpG sites on CpG group 1 region in panel B and on CpG island 2 in (**C**). *Ht* and *Br* promoters are represented by a gray box, whereas black box represents the relative position of Ht-RE1 sequence. Vertical black lines indicate locations of each CpG site. Bottom: Bisulfite sequencing patterns of the "CpG group 1" region (**B**) and CpG island 2 (**C**). Black filled circles and open circles represent methylated and nonmethylated CpG sites, respectively. Gray filled circles represent no CpG at the expected site. Each row represents a single clone, whereas each column represents a single CpG site. Percentage of methylated CpG sites in each group was calculated as mean percentage of methylation on 20 clones. **P*≤0.05 vs sham-operated animals, by Mann–Whitney *U* test. *Br* indicates brain promoter; *Ht*, heart promoter; NCX1, sodium/calcium exchanger 1; RE1, repressor element 1; and tMCAO, transient middle cerebral artery occlusion.

conserved sequence suggests that this binding site might exert an important role to regulate *Ncx1* transcription. Moreover, our results demonstrated that the transcriptional repressor REST downregulates *Ncx1* gene by recruiting 2 epigenetic modifiers mainly expressed in neurons, DNMT1 and MeCP2, on *Ht* sequence.[37] Importantly, when DNMT1 and MeCP2 are knocked down or overexpressed, an increase or a reduction in *Ht* promoter activity was found, respectively. In addition, the reduction of *Ncx1* gene products mediated by DNMT1 and MeCP2 overexpression was dependent on DNA methylation of *Ht* promoter. Indeed, *Ht* promoter activity was increased when neurons were exposed to the DNA demethylating agent 5-Aza but not with HDAC class I inhibitor MS275. On the other hand, MS275 but not 5-Aza upregulated *Br* promoter activity. Notably, the treatment with 5-Aza (Figure S1B) and the transfection of siRNAs against DNMT1 and MeCP2 activate *Ncx1-Ht* promoter in cortical neurons (Figure 2C) but not in U87 glial cell line (Figure S1C).

In light of these results, we could speculate that the epigenetic mechanism by which REST reduced *Ncx1* gene transcription via *Ht* promoter occurred by DNA methylation and is selectively orchestrated by DNMT1 and MeCP2 in neurons. In contrast, *Br* promoter-dependent *Ncx1* gene transcription is specifically regulated via the epigenetic erasers HDAC1 and HDAC2, as previously reported.[3]

Interestingly, also at the pathophysiological level REST downregulated *Ncx1* through 2 different mechanisms involving both *Br* and *Ht* promoters at different time points after stroke. Indeed, REST protein levels increased at 12 and 24 hours after cerebral ischemia, concomitantly with the downregulation of *Ncx1* mRNA and protein. Notably, at 12 hours after stroke, REST bound only the *Br* promoter, with HDAC1/HDAC2 complex,[3] whereas at 24 hours after tMCAO, REST bound selectively the *Ht* promoter with a new identified complex including DNMT1/MeCP2 (Figure 7).

The fact that HDAC1 and HDAC2 are bound to the *Ncx1-Br* promoter only 12 hours after ischemia and are absent after 24 hours confirms that histone deacetylation of *Ncx1-Br* is a time-limited event that does not shape the activity of the *Ncx1-Ht*. Moreover, it could be also hypothesized that at 12 and 24 hours of tMCAO different biochemical mechanisms are activated (such as phosphorylation, ubiquitination, and sumoylation), modulating the HDAC1/HDAC2 and DNMT1/MeCP2 protein levels and binding on *Ncx1-Br* and *Ncx1-Ht* promoters, respectively. Furthermore, ChIP experiments demonstrated that the silencing of HDAC1 and HDAC2 does not modify the enrichment of DNMT1 and MeCP2 on *Ncx1-Ht* promoter after 24 hours of tMCAO (Figure S4A and S4B). Collectively, these results indicate that under ischemic conditions deacetylation of *Ncx1-Br* and DNA methylation *of Ncx1-Ht* are not mutually exclusive. In light of these considerations, we can speculate that the increase in REST protein starts at 12 hours after tMCAO and, by recruiting HDAC1 and HDAC2, REST binds the ncx1-Br promoter sequence, thus determining the block of the *Ncx1-Br* dependent-transcription by histone deacetylation. However, in a later phase, our results suggest that REST/DNMT1/MeCP2 complex becomes more important for maintaining the downregulation of NCX1 mRNA, becuase the silencing of either DNMT1 or MeCP2 at 24 hours after stroke reverts the reduction of NCX1. These results are in accordance with other epigenetic phenomena regulated by the balance between deacetylation of histone tails and DNA methylation of a specific promoter sequence.[38]

In addition, we also found a significant increase in the methylation status of a group of 16 CpG sites within *Ht* promoter, including the *Ht*-RE1 site, at 24 hours after tMCAO.

Based on these results, we could speculate that *Ncx1* is downregulated by REST through the activation of 2 different epigenetic mechanisms after stroke. In particular, in the early phase of stroke, REST recruits epigenetic erasers, such as HDACs, to deacetylate histones on *Br* promoter, whereas in the later phase, REST recruits the epigenetic reader MeCP2 and the writer DNMT1 to hypermethylate the CpG group 1 including *Ht* promoter.

More important, silencing of each member of the DNMT1/MeCP2/REST complex, or the treatment with

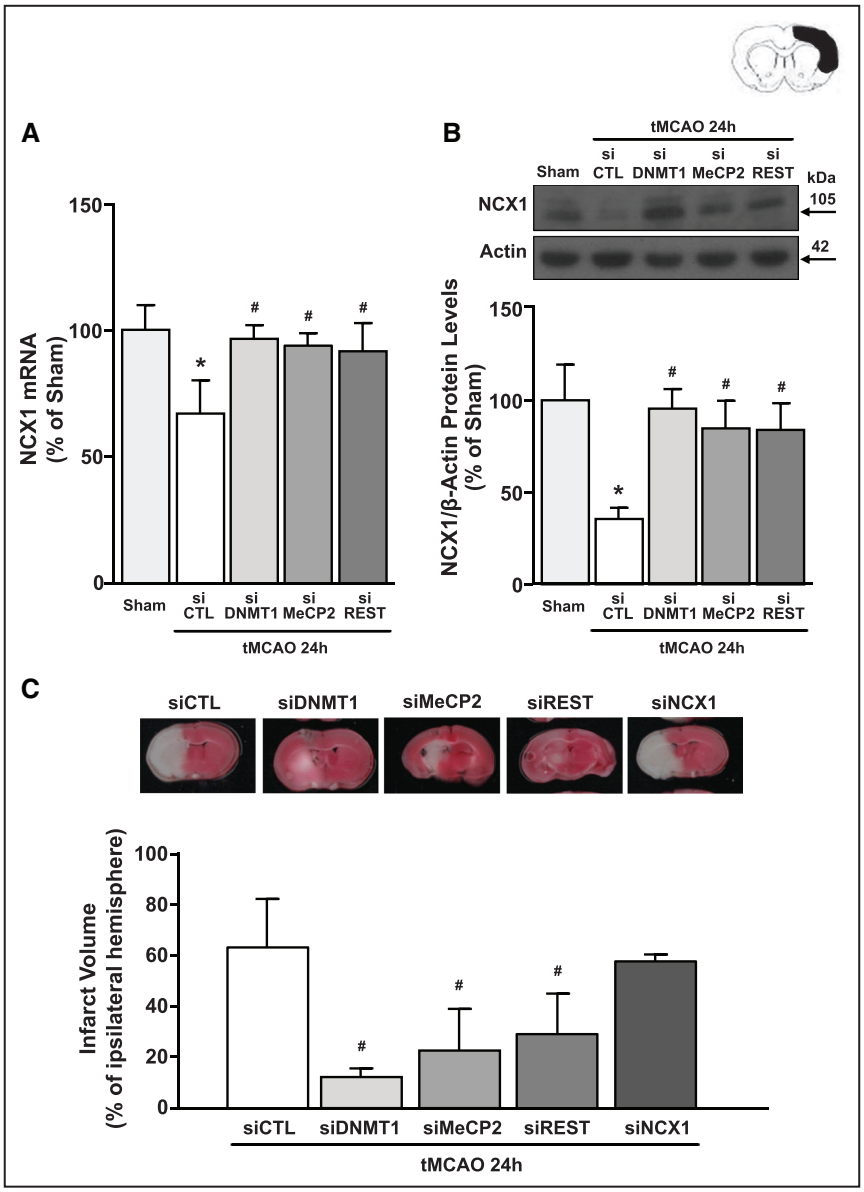


Figure 6. The knockdown of DNMT1, MeCP2, and REST counteracts the stroke-mediated downregulation of *Ncx1* mRNA and protein levels and ameliorates the ischemic damage in mice.

A and **B**, Effect of intracerebroventricular injections of siRNA for DNMT1, MeCP2, and REST on *Ncx1* mRNA and protein expression levels in mice subjected to tMCAO and euthanized after 24 hours. **P*≤0.05 vs Sham, #*P*≤0.05 vs tMCAO 24 h+siCTL by 1-way ANOVA analysis followed by Tukey's post hoc test (n=4). **C**, % of brain infarct volume in mice subjected to tMCAO after intracerebroventricular administration of siCTL, siDNMT1, siMeCP2, siREST, and siNCX1. Mice were euthanized 24 hours after tMCAO. Representative brain slices from each experimental group are shown on the top. #*P*≤0.05 vs tMCAO 24 h+siCTL, by 1-way ANOVA, followed by Newman–Keuls test (n=4). CTL indicates control; DNMT1, DNA-methyltransferase-1; MeCP2, methyl-CpG binding protein 2; NCX1, sodium/calcium exchanger 1; REST, repressor element 1-silencing transcription factor; si, small interfering; and tMCAO, transient middle cerebral artery occlusion.

the demethylating agent 5-Aza, reduced OGD/reoxygenation damage of primary cortical neurons by preventing *Ncx1* downregulation. Furthermore, *Ncx1* knocking-down counteracted the neuroprotective effect of siDNMT1, siMeCP2, siREST, and 5-Aza after OGD/reoxygenation, thus supporting the tight relationship between DNMT1/MeCP2/REST and *Ncx1* expression levels in the pathophysiological processes underlying stroke.

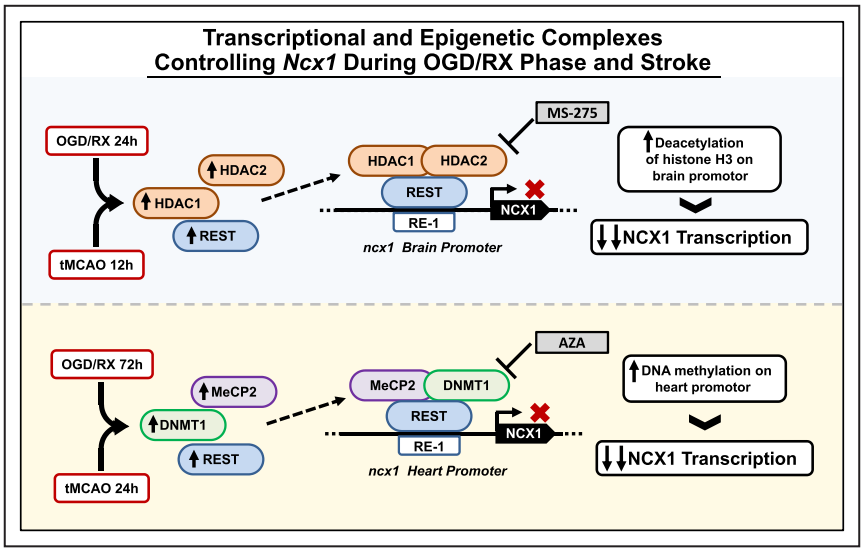


Figure 7. Schematic Illustration of transcriptional and epigenetic complexes controlling Ncx1 during OGD/reoxygenation phase and stroke.
The transcriptional factor REST modulates *Ncx1* through 2 different mechanisms involving *Br* and *Ht* at different time points after stroke. AZA indicates azacytidine; *Br*, brain promoter; DNMT1, DNA-methyltransferase-1; HDAC, histone deacetylase; *Ht*, heart promoter; MeCP2, methyl-CpG binding protein 2; NCX1, sodium/calcium exchanger 1; OGD, oxygen and glucose deprivation; RE1, repressor element 1; REST, repressor element 1-silencing transcription factor; and tMCAO, transient middle cerebral artery occlusion.

Accordingly, similar results were obtained in peri-ischemic temporoparietal cortex of mice subjected to tMCAO. In particular, either siREST, siDNMT1, or siMeCP2 intracerebroventricularly injected significantly (1) prevented *Ncx1* downregulation and (2) reduced the infarct size 24 hours after tMCAO, a time point in which the REST/DNMT1/MeCP2 complex is present and represses *Ncx1*, whereas REST/HDAC1/HDAC2 complex is absent and cannot modulate *Ncx1* transcription.

It is worth mentioning that MeCP2, DNMT1, and REST were already described in neuronal death processes. Indeed, elevated MeCP2 protein levels cause neurodegeneration in mice[39] and promote neuronal death in cortical neurons.[15] Moreover, it has been found that stroke reduced MeCP2 protein levels and its overexpression inhibits ischemic neuronal injury in hippocampi of ischemic mice.[40] On the contrary, other authors demonstrated that MeCP2 was reduced by stroke induced by MCAO model in mice. However, it should be highlighted that contrarily from our paper, in the study of Meng et al, the expression of MeCP2 was evaluated not in the penumbral vital tissue but only in the hippocampus and the occlusion of the MCA was maintained longer, thus generating a bigger infarct and a more extended ischemic core.[40] On the other hand, stroke upregulates DNMT1 in the brain, determining neuronal death as a consequence of DNA hypermethylation.[41] Furthermore, DNMT1 genetic or pharmacological inhibition determines a significant reduction in the infarct size and an improvement in neurological score and functional capacity after brain ischemia.[42,43]

In addition, different groups have demonstrated the neurodetrimental role of REST in in vitro and in vivo models of stroke[16,44,45] and that siREST intracerebroventricular treatment in ischemic mice, by reducing apoptotic neuronal death, improves motor function recovery up to 7 days of reperfusion.[45]

CONCLUSIONS

In summary, we identified a new epigenetic mechanism by which REST downregulates *Ncx1* after stroke. Specifically, we have found that the transcriptional repressor REST, by recruiting DNMT1 and MeCP2, reduces NCX1 in the cortex through the binding of its *Ht* promoter sequence. Therefore, these data allow the hypothesis that the inhibition of DNMT1/MeCP2/REST complex or DNA methylation of *Ncx1-Ht* promoter might represent a new possible pharmacological strategy to ameliorate brain ischemia.

ARTICLE INFORMATION

Received July 6, 2023; accepted January 3, 2024.

Affiliations

Division of Pharmacology, Department of Neuroscience, Reproductive and Dentistry Sciences, School of Medicine, "Federico II" University of Naples, Naples, Italy (N.G., A.S., L.S., L.M., O.C., P.B., P.M., G.P., L.F.); Institute of

Genetics and Biophysics "Adriano Buzzati Traverso", National Research Council of Italy, Napoli, Italy (S.F., D.M., F.D.R.); Department of Science and Technology, University of Sannio, Benevento, Italy (S.A.); and SynLab SDN IRCSS, Naples, Italy (L.A.).

Acknowledgments

N.G., P.M, L.A., and L.F participated in research design. All authors conducted experiments, and all authors performed data analysis and interpretation. N.G., L.A., and L.F drafted the article. L.F. supervised the study, and all authors read and approved the final article. Furthermore, we thank Dr Lucia d'Esposito and Massimiliano Cacace for their invaluable support in all in vivo procedures.

Sources of Funding

This work was supported by the following grants: Italian Ministry of Research MIUR Programma Operativo Nazionale (PON_01602, PON03PE_00146_1, PON PERMEDNET ArSol-1226) to L.A.; PRIN 2017 (2017 JL8SRX) to L.F.; PRIN 2017 (2017WJZ9W9_004) to P.M.; PRIN 2015 (2015BEX2BR_002) to F.D.R.; and MNESYS 2023 (PE0000006) by MIUR and National Recovery and Resilience Plan (NRRP) to L.F. and L.A.

Disclosures

None.

Supplemental Material

Figures S1–S7

REFERENCES

1. Molinaro P, Sirabella R, Pignataro G, Petrozziello T, Secondo A, Boscia F, Vinciguerra A, Cuomo O, Philipson KD, De Felice M, et al. Neuronal NCX1 overexpression induces stroke resistance while knockout induces vulnerability via Akt. *J Cereb Blood Flow Metab*. 2016;36:1790–1803. doi: 10.1177/0271678X15611913
2. Annunziato L, Pignataro G, Di Renzo GF. Pharmacology of brain Na+/Ca2+ exchanger: from molecular biology to therapeutic perspectives. *Pharmacol Rev*. 2004;56:633–654. doi: 10.1124/pr.56.4.5
3. Formisano L, Guida N, Valsecchi V, Cantile M, Cuomo O, Vinciguerra A, Laudati G, Pignataro G, Sirabella R, Di Renzo G, et al. Sp3/REST/HDAC1/HDAC2 complex represses and Sp1/HIF-1/p300 complex activates ncx1 gene transcription, in brain ischemia and in ischemic brain preconditioning, by epigenetic mechanism. *J Neurosci*. 2015;35:7332–7348. doi: 10.1523/JNEUROSCI.2174-14.2015
4. Ng GY, Lim YA, Sobey CG, Dheen T, Fann DY, Arumugam TV. Epigenetic regulation of inflammation in stroke. *Ther Adv Neurol Disord*. 2018;11:1–30. doi: 10.1177/1756286418771815
5. Rhee I, Bachman KE, Park BH, Jair KW, Yen RW, Schuebel KE, Cui H, Feinberg AP, Lengauer C, Kinzler KW, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*. 2002;416:552–556. doi: 10.1038/416552a
6. Carouge D, Host L, Aunis D, Zwiller J, Anglard P. CDKL5 is a brain MeCP2 target gene regulated by DNA methylation. *Neurobiol Dis*. 2010;38:414–424. doi: 10.1016/j.nbd.2010.02.014
7. Amir RE, Van den Veyver IB, Wan M, Tran CQ, Francke U, Zoghbi HY. Rett syndrome is caused by mutations in X-linked MECP2, encoding methyl-CpG-binding protein 2. *Nat Genet*. 1999;23:185–188. doi: 10.1038/13810
8. Della Ragione F, Vacca M, Fioriniello S, Pepe G, D'Esposito M. MECP2, a multi-talented modulator of chromatin architecture. *Brief Funct Genomics*. 2016;15:420–431. doi: 10.1093/bfgp/elw023
9. Horvath PM, Monteggia LM. MeCP2 as an activator of gene expression. *Trends Neurosci*. 2018;41:72–74. doi: 10.1016/j.tins.2017.11.005
10. Lombardi LM, Baker SA, Zoghbi HY. MECP2 disorders: from the clinic to mice and back. *J Clin Invest*. 2015;125:2914–2923. doi: 10.1172/jci78167
11. Paonessa F, Latifi S, Scarongella H, Cesca F, Benfenati F. Specificity protein 1 (Sp1)-dependent activation of the synapsin I gene (SYN1) is modulated by RE1-silencing transcription factor (REST) and 5′-cytosine-phosphoguanine (CpG) methylation. *J Biol Chem*. 2013;288:3227–3239. doi: 10.1074/jbc.M112.399782
12. Ballas N, Mandel G. The many faces of REST oversee epigenetic programming of neuronal genes. *Curr Opin Neurobiol*. 2005;15:500–506. doi: 10.1016/j.conb.2005.08.015
13. Jung BP, Jugloff DG, Zhang G, Logan R, Brown S, Eubanks JH. The expression of methyl CpG binding factor MeCP2 correlates with cellular differentiation in the developing rat brain and in cultured cells. *J Neurobiol*. 2003;55:86–96. doi: 10.1002/neu.10201
14. Ramocki MB, Tavyev YJ, Peters SU. The MECP2 duplication syndrome. *Am J Med Genet A*. 2010;152a:1079–1088. doi: 10.1002/ajmg.a.33184
15. Dastidar SG, Bardai FH, Ma C, Price V, Rawat V, Verma P, Narayanan V, D'Mello SR. Isoform-specific toxicity of Mecp2 in postmitotic neurons: suppression of neurotoxicity by FoxG1. *J Neurosci*. 2012;32:2846–2855. doi: 10.1523/jneurosci.5841-11.2012
16. Noh KM, Hwang JY, Follenzi A, Athanasiadou R, Miyawaki T, Greally JM, Bennett MV, Zukin RS. Repressor element-1 silencing transcription factor (REST)-dependent epigenetic remodeling is critical to ischemia-induced neuronal death. *Proc Natl Acad Sci U S A*. 2012;109:E962–E971. doi: 10.1073/pnas.1121568109
17. Dock H, Theodorsson A, Theodorsson E. DNA methylation inhibitor zebularine confers stroke protection in ischemic rats. *Transl Stroke Res*. 2015;6:296–300. doi: 10.1007/s12975-015-0397-7
18. Scheller T, Kraev A, Skinner S, Carafoli E. Cloning of the multipartite promoter of the sodium-calcium exchanger gene NCX1 and characterization of its activity in vascular smooth muscle cells. *J Biol Chem*. 1998;273:7643–7649. doi: 10.1074/jbc.273.13.7643
19. Formisano L, Guida N, Valsecchi V, Pignataro G, Vinciguerra A, Pannaccione A, Secondo A, Boscia F, Molinaro P, Sisalli MJ, et al. NCX1 is a new rest target gene: role in cerebral ischemia. *Neurobiol Dis*. 2013;50:76–85. doi: 10.1016/j.nbd.2012.10.010
20. Li H, Rauch T, Chen ZX, Szabó PE, Riggs AD, Pfeifer GP. The histone methyltransferase SETDB1 and the DNA methyltransferase DNMT3A interact directly and localize to promoters silenced in cancer cells. *J Biol Chem*. 2006;281:19489–19500. doi: 10.1074/jbc.M513249200
21. Tillotson R, Selfridge J, Koerner MV, Gadalla KKE, Guy J, De Sousa D, Hector RD, Cobb SR, Bird A. Radically truncated MeCP2 rescues Rett syndrome-like neurological defects. *Nature*. 2017;550:398–401. doi: 10.1038/nature24058
22. Formisano L, Laudati G, Guida N, Mascolo L, Serani A, Cuomo O, Cantile M, Boscia F, Molinaro P, Anzilotti S, et al. HDAC4 and HDAC5 form a complex with DREAM that epigenetically down-regulates NCX3 gene and its pharmacological inhibition reduces neuronal stroke damage. *J Cereb Blood Flow Metab*. 2020;40:2081–2097. doi: 10.1177/0271678X19884742
23. Buira SP, Albasanz JL, Dentesano G, Moreno J, Martín M, Ferrer I, Barrachina M. DNA methylation regulates adenosine a(2A) receptor cell surface expression levels. *J Neurochem*. 2010;112:1273–1285. doi: 10.1111/j.1471-4159.2009.06538.x
24. Marutha Ravindran CR, Ticku MK. Effect of 5-azacytidine on the methylation aspects of NMDA receptor NR2B gene in the cultured cortical neurons of mice. *Neurochem Res*. 2009;34:342–350. doi: 10.1007/s11064-008-9783-9
25. Sanguigno L, Guida N, Anzilotti S, Cuomo O, Mascolo L, Serani A, Brancaccio P, Pennacchio G, Licastro E, Pignataro G, et al. Stroke by inducing HDAC9-dependent deacetylation of HIF-1 and Sp1, promotes TfR1 transcription and GPX4 reduction, thus determining ferroptotic neuronal death. *Int J Biol Sci*. 2023;19:2695–2710. doi: 10.7150/ijbs.80735
26. Percie du Sert N, Hurst V, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, et al. The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *BMJ Open Sci*. 2020;4:e100115. doi: 10.1136/bmjos-2020-100115
27. Longa EZ, Weinstein PR, Carlson S, Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. *Stroke*. 1989;20:84–91. doi: 10.1161/01.STR.20.1.84
28. Pignataro G, Meller R, Inoue K, Ordonez AN, Ashley MD, Xiong Z, Gala R, Simon RP. In vivo and in vitro characterization of a novel neuroprotective strategy for stroke: ischemic postconditioning. *J Cereb Blood Flow Metab*. 2008;28:232–241. doi: 10.1038/sj.jcbfm.9600559
29. Formisano L, Guida N, Mascolo L, Serani A, Laudati G, Pizzorusso V, Annunziato L. Transcriptional and epigenetic regulation of ncx1 and ncx3 in the brain. *Cell Calcium*. 2020;87:102194. doi: 10.1016/j.ceca.2020.102194
30. Guida N, Laudati G, Serani A, Mascolo L, Molinaro P, Montuori P, Di Renzo G, Canzoniero LMT, Formisano L. The neurotoxicant PCB-95 by increasing the neuronal transcriptional repressor REST down-regulates caspase-8 and increases Ripk1, Ripk3 and MLKL expression determining necroptotic neuronal death. *Biochem Pharmacol*. 2017;142:229–241. doi: 10.1016/j.bcp.2017.06.135

31. Valsecchi V, Pignataro G, Del Prete A, Sirabella R, Matrone C, Boscia F, Scorziello A, Sisalli MJ, Esposito E, Zambrano N, et al. NCX1 is a novel target gene for hypoxia-inducible factor-1 in ischemic brain preconditioning. *Stroke*. 2011;42:754–763. doi: 10.1161/STROKEAHA.110.597583
32. Koban MU, Brugh SA, Riordon DR, Dellow KA, Yang HT, Tweedie D, Boheler KR. A distant upstream region of the rat multipartite Na(+)-Ca(2+) exchanger NCX1 gene promoter is sufficient to confer cardiac-specific expression. *Mech Dev*. 2001;109:267–279. doi: 10.1016/s0925-4773(01)00548-2
33. Cao H, Wang L, Chen B, Zheng P, He Y, Ding Y, Deng Y, Lu X, Guo X, Zhang Y, et al. DNA demethylation upregulated Nrf2 expression in Alzheimer's disease cellular model. *Front Aging Neurosci*. 2015;7:244. doi: 10.3389/fnagi.2015.00244
34. Hwang JY, Zukin RS. REST, a master transcriptional regulator in neurodegenerative disease. *Curr Opin Neurobiol*. 2018;48:193–200. doi: 10.1016/j.conb.2017.12.008
35. Bryne JC, Valen E, Tang MH, Marstrand T, Winther O, da Piedade I, Krogh A, Lenhard B, Sandelin A. JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic Acids Res*. 2008;36:D102–D106. doi: 10.1093/nar/gkm955
36. Müller JG, Isomatsu Y, Koushik SV, O'Quinn M, Xu L, Kappler CS, Hapke E, Zile MR, Conway SJ, Menick DR. Cardiac-specific expression and hypertrophic upregulation of the feline Na(+)-Ca(2+) exchanger gene H1-promoter in a transgenic mouse model. *Circ Res*. 2002;90:158–164. doi: 10.1161/hh0202.103231
37. Shahbazian MD, Antalffy B, Armstrong DL, Zoghbi HY. Insight into Rett syndrome: MeCP2 levels display tissue- and cell-specific differences and correlate with neuronal maturation. *Hum Mol Genet*. 2002;11:115–124. doi: 10.1093/hmg/11.2.115
38. Borrelli E, Nestler EJ, Allis CD, Sassone-Corsi P. Decoding the epigenetic language of neuronal plasticity. *Neuron*. 2008;60:961–974. doi: 10.1016/j.neuron.2008.10.012
39. Montgomery KR, Louis Sam Titus ASC, Wang L, D'Mello SR. Elevated MeCP2 in mice causes neurodegeneration involving tau dysregulation and Excitotoxicity: implications for the understanding and treatment of MeCP2 triplication syndrome. *Mol Neurobiol*. 2018;55:9057–9074. doi: 10.1007/s12035-018-1046-4
40. Meng L, Feng B, Luan L, Fang Z, Zhao G. MeCP2 inhibits ischemic neuronal injury by enhancing methylation of the FOXO3a promoter to repress the SPRY2-ZEB1 axis. *Exp Mol Med*. 2022;54:1076–1085. doi: 10.1038/s12276-022-00790-4
41. Endres M, Meisel A, Biniszkiewicz D, Namura S, Prass K, Ruscher K, Lipski A, Jaenisch R, Moskowitz MA, Dirnagl U. DNA methyltransferase contributes to delayed ischemic brain injury. *J Neurosci*. 2000;20:3175–3181. doi: 10.1523/jneurosci.20-09-03175.2000
42. Endres M, Fan G, Meisel A, Dirnagl U, Jaenisch R. Effects of cerebral ischemia in mice lacking DNA methyltransferase 1 in post-mitotic neurons. *Neuroreport*. 2001;12:3763–3766. doi: 10.1097/00001756-200112040-00032
43. Mondal NK, Behera J, Kelly KE, George AK, Tyagi PK, Tyagi N. Tetrahydrocurcumin epigenetically mitigates mitochondrial dysfunction in brain vasculature during ischemic stroke. *Neurochem Int*. 2019;122:120–138. doi: 10.1016/j.neuint.2018.11.015
44. Formisano L, Noh K, Miyawaki T, Mashiko T, Bennett M, Zukin R. Ischemic insults promote epigenetic reprogramming of mu opioid receptor expression in hippocampal neurons. *Proc Natl Acad Sci USA*. 2007;104:4170–4175. doi: 10.1073/pnas.0611704104
45. Morris-Blanco KC, Kim T, Bertogliat MJ, Mehta SL, Chokkalla AK, Vemuganti R. Inhibition of the epigenetic regulator REST ameliorates ischemic brain injury. *Mol Neurobiol*. 2019;56:2542–2550. doi: 10.1007/s12035-018-1254-y