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The gene silencing transcription factor REST represses miR-132 expression in hippocampal neurons destined to die

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Abstract

The gene silencing transcription factor REST/NRSF (Repressor Element-1 (RE1) Silencing Transcription Factor/Neuron-Restrictive Silencer Factor) actively represses a large array of coding and noncoding neuron-specific genes important to synaptic plasticity including miR-132. miR-132 is a neuron-specific microRNA and plays a pivotal role in synaptogenesis, synaptic plasticity and structural remodeling. However, a role for miR-132 in neuronal death is not, as yet, well-delineated. Here we show that ischemic insults promote REST binding and epigenetic remodeling at the miR-132 promoter and silencing of miR-132 expression in selectively-vulnerable hippocampal CA1 neurons. REST occupancy was not altered at the miR-9 or miR-124a promoters despite the presence of RE1 sites, indicating REST target specificity. Ischemia induced a substantial decrease in two marks of active gene transcription, dimethylation of lysine 4 on core histone 3 (H3K4me2) and acetylation of lysine 9 on H3 (H3K9ac) at the miR-132 promoter. RNAi-mediated depletion of REST *in vivo* blocked ischemia-induced loss of miR-132 in insulted hippocampal neurons, consistent with a causal relation between activation of REST and silencing of miR-132. Overexpression of miR-132 in primary cultures of hippocampal neurons or delivered directly into the CA1 of living rats by means of the lentiviral expression system prior to induction of ischemia afforded robust protection against ischemia-induced neuronal death. These findings document a previously unappreciated role for REST-dependent repression of miR-132 in the neuronal death associated with global ischemia and identify a novel therapeutic target for amelioration of the neurodegeneration and cognitive deficits associated with ischemic stroke.

Keywords

microRNAs; epigenetic modifications; global ischemia; neuronal death; hippocampus

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INTRODUCTION

REST (Repressor Element-1 Silencing Transcription factor, also termed NRSF) is a gene-silencing transcription factor that is widely expressed during embryogenesis and the late stages of neuronal differentiation and its loss is critical for elaboration of the neuronal phenotype [1–4]. In pluripotent stem cells and neural progenitors, REST actively represses a large array of coding and noncoding neuron-specific genes important to synaptic plasticity and structural remodeling including miR-132 [1–4]. A subset of microRNAs including miR-9, miR-124a, and miR-132 contain RE-1 sites within their promoter regions in mouse and humans [5,6] and are functionally validated targets of REST in mammalian cell lines [5]. In mature neurons, REST is quiescent, but can be reactivated in selectively-vulnerable hippocampal neurons by ischemic insults [7–9] and seizures [10,11] and in human brain by normal aging [12]. In Huntington's disease, REST aberrantly accumulates in the nucleus of medium aspiny striatal neurons [13]. However, whether neuronal insults promote activation and recruitment of REST to the promoters of miRNAs and whether REST orchestrates epigenetic remodeling and silencing of miRNAs in response to neuronal insults is, as yet, unclear.

microRNAs (miRNAs) are small, non-coding RNAs that serve to negatively regulate protein expression through mRNA degradation and translational repression [14,15]. miRNAs are particularly enriched in the nervous system, where they play a pivotal role in synaptic plasticity and structural remodeling [16]. Emerging evidence points to a widespread role for miRNAs as key modulators of target gene expression in neurons. microRNAs play a pivotal role in synapse formation and maturation and dendritogenesis in early stages of brain development [4,14,15]. A key feature of neuronal miRNAs is their ability to regulate entire networks of nonneuronal genes in an activity- or experience-dependent manner [15]. Moreover, neuron-specific miRNAs can regulate mRNA translation locally in dendrites, where they are thought to play an important role in activity-dependent synaptic plasticity [4,14,15].

miR-132 is one of the best characterized miRNAs in the nervous system. In neurons, miR-132 localizes to the presynaptic membrane, where it represses methyl CpG binding protein-2 (MeCP2), a gene-silencing factor that recognizes methylated DNA and reads epigenetic marks [17,18]. Repression of MeCP2 results in activation of brain-derived neurotrophic factor (BDNF), which in turn activates miR-132, thereby constituting a self-regulatory mechanism [19]. miR-132 is well-known for its role in plasticity and activity-dependent shaping of dendritic morphology and synaptic function [14,15]. miR-132 is important for activity-dependent dendritic growth and remodeling, implicating miR-132 in the development and fine-tuning of neural circuits in response to environmental cues [20]. An important mechanism by which miR-132 exerts its growth-promoting effects is by repression of small Rho GTPases, such as p250GAP, which serve as regulators of the dendritic actin cytoskeleton. Suppression of p250GAP by miR-132 is required for both basal and activity-induced dendrite growth [20–22]. Dysregulation of miR-132 and its target proteins are implicated in the pathophysiology of epilepsy [23,24], Alzheimer's disease [25,26], and Parkinson's disease [27]. Whereas a role for miR-132 in the neuronal death

associated with these disorders is well-established [14], its role in the pathogenesis of global ischemia is, as yet, unclear.

Transient global ischemia arising as a consequence of cardiac arrest in humans or induced experimentally in animals causes selective, delayed death of hippocampal CA1 pyramidal neurons and cognitive impairment [28–30]. The long delay between ischemic insult and neuronal death is consistent with a role for gene transcription. Effective treatments to ameliorate the neurodegeneration and cognitive dysfunction associated with global ischemia are an unmet need. The present study was undertaken to examine a possible role for miR-132, a neuron-specific miRNA known to play a pivotal role in brain development, in ischemia-induced neuronal death. Our findings demonstrate that ischemic insults trigger activation of REST and REST-dependent silencing of miR-132 in selectively-vulnerable CA1 neurons and that REST-dependent repression of miR-132 is critical to ischemia-induced neuronal death in a clinically-relevant model of ischemic stroke *in vivo*.

RESULTS

Ischemia activates REST, recruits REST to the miR-132 promoter, and silences miR-132 in CA1

Transient global ischemia in rats is a well-established model of neuronal insult in which cell death occurs primarily in CA1 pyramidal neurons and is delayed by 3–4 d, allowing examination of molecular mechanisms that underlie cell death [28–30]. Global ischemia induced a marked increase in REST protein abundance in the nuclear fraction of the selectively vulnerable CA1, evident at 24 h and 48 h (Fig.1a). Ischemia induced enrichment of REST in close proximity to the three RE1 sites within the miR-132 promoter of rat identified by the JASPAR CORE database (http://jaspar.genereg.net/), relative to that of sham-operated (control) animals (Fig. 1b). To examine whether the increase in REST at the miR-132 promoter translates into altered miR-132 expression, we performed RT-qPCR at times after ischemia. Ischemia induced a marked decrease in miR-132 expression in CA1, evident at 24 h and 48 h after ischemia (Fig. 1c). Whereas ischemia induced enrichment for REST at the miR-132 promoter and repressed miR-132 expression, it did not significantly alter occupancy of REST at the promoters of miR-9 or miR-124a (each of which contain one or more RE1 sites), nor did it alter miR-9 or miR-124a expression (Fig. 1d–g). Moreover, ischemia did not detectably alter REST occupancy at sites either 8.5 kb upstream or 10 kb downstream from the miR-132 promoter (negative control sites) or at the promoter of the β-actin gene, a negative control (data not illustrated). Furthermore, the expression of miR-132, miR-9 and miR-124a was not altered in the resistant CA3 (Fig. 1h). These data indicate that global ischemia promotes REST abundance and REST binding to the miR-132 promoter in the selectively vulnerable CA1 *in vivo* in a target- and subfield-specific manner.

REST orchestrates epigenetic remodeling of the miR-132 promoter

The findings thus far indicate that REST regulates miR-132 expression in insulted hippocampal neurons, but do not address the mechanism by which this occurs. To address this issue, we examined alterations in epigenetic marks proximal to the RE1 sites in the promoter region of miR-132 (Fig. 2a, *upper*). Toward this end, we examined dimethylation

of lysine 4 on histone 3 (H3K4me2), acetylation of lysine 9 on histone 3 (H3K9ac), epigenetic marks of open chromatin and active gene transcription, and trimethylation of lysine 27 on histone (H3K27me3), an epigenetic mark of gene repression, in the selectively vulnerable CA1 after ischemia. Whereas H3K4 is demethylated by LSD1, H3K9 is deacetylated by HDACs. Global ischemia induced a marked decrease in H3K4me2 in the CA1, evident at 24 h and 48 h (Fig. 2a, *lower*), and a marked decrease in H3K9ac in the CA1, evident at 48 h (Fig. 2b) after ischemia at the promoter of miR-132 as assessed by ChIP-qPCR. In contrast, ischemia did not detectably alter methylation of H3K4 or acetylation of H3K9 at sites 8.5 kb upstream or 10 kb downstream of the miR-132 promoter (Fig. 2a,b). Moreover, ischemia did not significantly alter methylation of H3K27 at the promoter of miR-132 (Fig. 2c). These findings indicate that the REST corepressor complex is active and orchestrates epigenetic remodeling of the REST target miR-132 in CA1 neurons destined to die

Knockdown of REST prevents the ischemia-induced decrease in miR-132

The results thus far indicate a possible role of REST in miR-132 expression, but do not address the causal relation between activation of REST and silencing of miR-132 regulation. To address this issue, we designed and validated two REST shRNA constructs that effectively suppress REST expression in primary cultures of hippocampal neurons (Fig. 3a,b) and in the CA1 *in vivo* [9]. We next assessed the impact of acute knockdown of REST on miR-132 expression. Toward this end, we delivered lentivirus carrying REST shRNA unilaterally into the CA1 by stereotaxic injection, and 14 d later, subjected rats to global ischemia (Fig. 3c, *upper*). Whereas ischemia suppressed miR-132 expression in the CA1 of rats expressing NT-shRNA, miR-132 expression was not detectably altered in the CA1 of rats expressing REST shRNA as assessed by RT-qPCR, at 48 h after ischemia (Fig. 3c, *lower*). In contrast, ischemia did not detectably alter miR-124a expression in the CA1 of animals expressing either NT- or REST shRNA (Fig. 3d). These findings demonstrate that acute knockdown of REST prevents the ischemia-induced decrease in miR-132, consistent with the notion that REST is essential for the ischemia-induced decrease in miR-132.

Overexpression of miR-132 affords neuroprotection against ischemia-induced cell death

To address a possible causal relation between loss of miR-132 and ischemia-induced neuronal death, we overexpressed miR-132 in primary cultures of hippocampal neurons at *DIV*7, and 7d later, subjected neurons to OGD. OGD is a well-established model of global ischemia *in vitro*. Toward this end, we examined the impact of OGD on REST abundance and miR-132 expression. OGD elicited an increase in REST and a decrease in miR-132, but not miR-9 or miR-132, in primary cultures of hippocampal neurons *in vitro* (Fig. 4a,b). Next, we validated miR-132 overexpression in primary cultures of hippocampal neurons transduced by means of the lentivirus expression system. Lentiviral expression was robust in hippocampal neurons at 7 d after transduction, as assessed by GFP fluorescence (Fig. 4c, *left*). miR-132, but not miR-9 or miR-124a, was overexpressed ~10-fold relative to that of negative-miR expressing or nontransduced (control) neurons (Fig. 4c, *right*). Finally, we examined impact of miR-132 on ischemia-induced neuronal death *in vitro*. Overexpression of miR-132 markedly reduced OGD-induced neuronal death, as assessed by uptake of the DNA indicator dye propidium iodide (Fig. 4d).

To establish a causal relation between the decrease in miR-132 and ischemia-induced neuronal death in a clinically relevant model of ischemic stroke, we delivered lentivirus carrying miR-132 or negative miR by unilateral stereotaxic injection directly into the right CA1 of living animals; 14 d later, rats were subjected to global ischemia or sham operation. Whereas negative miR-expressing rats subjected to global ischemia exhibited robust neuronal death in the hippocampal CA1, as assessed by toluidine blue (Fig. 5a,b) and Fluoro-Jade (Fig. 5c,d) staining at 7 d after insult, neuronal death was strikingly reduced in the CA1 of rats overexpressing miR-132. Collectively, these findings indicate that miR-132 overexpression affords robust protection against global ischemia-induced neuronal death, and document a causal relation between the ischemia-induced loss of miR-132 and neuronal death in *in vitro* and *in vivo* models of ischemia.

DISCUSSION

In this study we show that activation of the gene silencing transcription factor REST is causally related to loss of miR-132 in postischemic CA1 neurons and that REST-dependent repression of miR-132 is critical to the neuronal death associated with global ischemia. miR-132 overexpression in hippocampal neurons or the CA1 of living rats affords robust protection against ischemia-induced neuronal death. To our knowledge, this is the first demonstration that regulation of miR-132 or any miRNA by REST is causally related to ischemia-induced neuronal death. Whereas miR-132 is implicated in the neuronal death associated with epilepsy [23,24], Alzheimer's disease [25,26] and Parkinson's disease [27] and is thought to play a role in ischemic preconditioning [31], a role for miR-132 in ischemia-induced neuronal death has remained unclear. We show that the repressor REST is enriched at the miR-132 promoter, where it orchestrates epigenetic remodeling and silencing of miR-132 expression in postischemic CA1 (but not CA3). Consistent with this, ischemia triggers a substantial decrease in two epigenetic marks of active gene transcription, H3K4me2 and H3K9ac (but not H3K27me3, a mark of gene repression), in the selectively vulnerable CA1 after ischemia. By contrast, neither REST occupancy at the miR-9 or miR-124a promoters nor miR-9 or miR-124a expression is altered in post ischemic neurons, despite the presence of RE1 sites, consistent with REST target specificity. Emerging evidence supports the concept that REST regulates different networks of target genes in a context-, age- and cell type-specific manner [9]. These findings document a previously unappreciated role for REST-dependent repression of miR-132 in the neuronal death associated with global ischemia and identify a novel therapeutic target for amelioration of the neurodegeneration and cognitive deficits associated with ischemic stroke.

A fundamental mechanism by which REST silences target genes is that of epigenetic remodeling [32]. REST binds the RE1 element of target genes and recruits C-terminal cofactor for REST (CoREST) [33,34], and mSin3A [35–37], corepressor platforms that recruit histone deacetylases (HDACs) 1 and 2. HDACs deacetylate core histone proteins and affect dynamic and reversible gene silencing [1,2,38]. In addition, REST recruits the site-specific histone protein G9a, which methylates lysine 9 of histone H3 (H3K9) [39,40], the site-specific histone demethylase LSD1, which removes methyl groups from mono- or dimethylated lysine 4 of core histone protein H3 (H3K4) [41,42], and MeCP2, a protein that reads epigenetic marks on core histones and hotspots of DNA methylation [39,43]. Recent

studies indicate that deacetylation of H3K9 by HDACs stimulates LSD activity, which in turn, removes methylation marks from H3K4 [2]. Our findings in the present study that H3K4me2 and H3K9ac, marks of active gene transcription, are reduced at the miR-132 promoter in postischemic CA1 are consistent with transcriptional repression. Whereas dimethylation of H3K4 is a functional read-out of LSD1, a reduction in acetylation of H3K9 is a functional read-out of HDAC activity. Our finding that H3K27me3, a mark of gene repression, is not altered at the miR-132 promoter is consistent with the possibility that the polycomb protein EZH1/2 is not recruited to the miR-132 promoter. EZH2, the catalytically active component of polycomb repressive complex 2 (PRC2), is a histone methyltransferase that confers a trimethylation mark on Lys27 of histone H3 (H3K27me3), a mark of gene repression [44–46]. Recent studies indicate that PRC2 is recruited by REST *via* HOTAIR [47] and that PRC1 interacts with REST at RE1 sites [48]. Whereas we show that REST is causally related to ischemia-induced loss of miR-132, we do not address a causal relation between REST and epigenetic remodeling of the miR-132 promoter.

An emerging view is that epigenetics includes DNA methylation, histone modifications and miRNA-dependent gene silencing [3,4,49,50]. Collectively, these mechanisms influence gene expression and phenotype. An interesting aspect of miRNA function is its ability to induce epigenetic modifications directly and indirectly [49]. miRNAs act indirectly to promote epigenetic remodeling by regulation of components of the epigenetic machinery including, but not limited to DNA methyltransferases, HDACs, polycomb group proteins and MeCP2, which in turn regulate the expression of a wide array of genes including miRNAs [51]. Thus, miRNAs can participate in ‘epigenetic-miRNA regulatory circuits’ [51]. As an example, miR-132 represses MeCP2, leading to activation of BDNF, which in turn functions as an activator of miR-132 [19].

Our finding that miR-132 is dysregulated in global ischemia is consistent with findings of others that miR-132 and its target proteins are implicated in the pathophysiology of other brain disorders including focal ischemia [31], epilepsy [23,24,52], Alzheimer’s disease [25,26] and Parkinson’s disease [27]. Whereas findings in the present study show that global ischemia (a paradigm that induces neuronal death) induces downregulation of miR-132 in the hippocampal CA1, findings of others [31] show that ischemic preconditioning (a paradigm that affords neuroprotection) induces downregulation of miR-132 in the cortex in a model of focal ischemia. However, upon closer inspection, the same study [31] reports that focal ischemia (which induces fulminating neuronal death primarily in the striatum and cortex) also elicits downregulation of miR-132 expression. Thus, the two models of ischemic stroke, focal and global ischemia, exert similar effects on miR-132 expression, despite the fact that they induce cell death *via* different mechanisms and target different populations of neurons. Studies involving unbiased, genome-wide studies and bioinformatic analysis reveal upregulation of miR-132 in the hippocampal CA3 of animals with status epilepticus and in humans with epilepsy [24,53,54]. Chemically-engineered oligonucleotides or ‘antagomirs’ directed against miR-132 efficiently silenced miR-132 and afforded neuroprotection in animal with status epilepticus, consistent with the concept that miR-132 is causally-related to seizure-induced neuronal death [24]. In contrast, in Alzheimer’s disease, miR-132 appears to have a pro-survival function. These studies show that miR-132

is downregulated, and its targets PTEN, P300, and FOXO3, are upregulated in the temporal cortical areas and hippocampal CA1 from humans with Alzheimer's disease [25,26]. The present study adds global ischemia to the growing list of brain disorders in which miR-132 is causally related to neuronal survival or neuronal death. We show that REST-dependent silencing of miR-132 is causally related to ischemia-induced neuronal death and that overexpression of miR-132 in the CA1 of living rats affords robust protection against ischemia-induced neuronal death in a clinically-relevant model of ischemic stroke.

Key targets of miR-132, including transcription factors and signaling molecules, are expressed in neurons and are implicated in neuronal death. In the present study, although we find that global ischemia elicits a marked upregulation of REST and a marked downregulation of miR-132, surprisingly we did not detect altered expression of several prominent targets of miR-132 implicated in other paradigms of neuronal death (MeCP2, PTEN, FOXO3a, DNMT3a, AchE) in the selectively-vulnerable CA1 at either 24 or 48 h after ischemia. Future studies are warranted to identify possible targets of miR-132 that are regulated by and/or contribute to global ischemia-induced neuronal death.

The present study was undertaken to examine a possible role for miR-132, a neuron-specific miRNA known to play a pivotal role in brain development, in ischemia-induced neuronal death. Our findings demonstrate that activation of REST is causally related to silencing of miR-132 in selectively-vulnerable CA1 neurons and that REST-dependent repression of miR-132 is critical to ischemia-induced neuronal death in a clinically-relevant model of ischemic stroke *in vivo*. Notably, overexpression of miR-132 in the hippocampal CA1 of living rats affords robust protection against ischemia-induced neuronal death. These findings document a previously unappreciated role for REST-dependent repression of miR-132 in the neuronal death associated with global ischemia and identify a novel therapeutic target for amelioration of the neurodegeneration and cognitive deficits associated with ischemia. These findings implicate miRNAs in the pathophysiology of global ischemia and point to a role for miRNA-132 as a novel therapeutic target to reduce the hippocampal injury associated with ischemic stroke.

MATERIALS & METHODS

Animals

Four to six-week-old male Sprague-Dawley rats weighing 150–200 gm (Charles River Laboratories, Inc.) were maintained in a temperature- and light-controlled environment with a 12:12 h light/dark cycle and were treated in accordance with the principles and procedures of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Protocols were approved by the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Primary cultures of hippocampal neurons and oxygen-glucose deprivation (OGD)

Primary cultures of hippocampal neurons were prepared from embryonic (E18) rats as described [9]. Cultures were maintained for 7 days *in vitro* (DIV) before transduction with lentiviral constructs. OGD was performed at 14 DIV. To induce OGD, neurons were

exposed to serum-free, glucose-free medium saturated with 95% N_2/5% CO_2 for 30 min at 37°C in an airtight, anoxic chamber (Billups-Rothenberg). Cultures were returned to oxygenated, glucose-containing medium under normoxic conditions for 24 h and were processed for western blotting analysis, qRT-PCR or uptake of propidium iodide to assess neuronal death.

Global ischemia

Animals were subjected to transient global ischemia by the four-vessel occlusion method as described [9,55]. For sham surgery, animals were subjected to the identical surgical procedure, except that the carotid arteries were not occluded. During recovery from anesthesia, body temperature was maintained at 37.5 ± 0.5 °C by external warming. Animals that failed to show complete loss of the righting reflex and dilation of the pupils 2 min after occlusion, and the rare animals that exhibited obvious behavioral manifestations (abnormal vocalization when handled, generalized convulsions, loss of > 20% body weight by 3–7 d or hypoactivity) were excluded from the study.

Western blots

Western blot analysis was performed as described [55–57]. In brief, neurons were washed with ice-cold PBS and lysed in buffer containing (in mM): 25 Tris-HCl, pH 7.4, 150 NaCl, 1 EDTA, 1× protease inhibitor cocktail (Sigma), 0.1% SDS and 0.5% Na deoxycholate. For the tissue samples, hippocampi were rapidly dissected and transverse slices of dorsal hippocampus (1 mm) were cut with a Mcllwain tissue chopper. The CA1 subfield was rapidly microdissected and lysed in buffer supplemented with a 1% cocktail of protease inhibitors (Sigma). Protein concentrations were measured by means of the bicinchoninic acid (BCA) assay (Pierce). Equal concentrations of protein (30 ~ 50 μg) were separated on 4–20% SDS-PAGE gels, transferred to nitrocellulose membranes, and incubated with anti-REST, 1:1000 (Millipore) and anti-β-actin, 1:50000(Sigma), followed by chemiluminescent detection(ECL; Amersham Biosciences). Band densities were normalized to β-actin. Mean band densities for samples from experimental animals were normalized to the corresponding samples from control neurons or sham animals.

Single-Locus ChIP-qPCR

The CA1 subfields were rapidly microdissected from hippocampi and used for the ChIP assay as described [57]. In brief, the CA1 was immersed in 1% formaldehyde for 30 min at room temperature to cross-link REST or histone protein to DNA and the cross-linking halted by addition of 0.125 M glycine. The samples were lysed and sonicated to generate fragments of chromosomal DNA in the range of 300 bp. Aliquots of chromatin were diluted with ChIP dilution buffer to a final volume of 1 ml, and an aliquot of pre-immunoprecipitated chromatin (“input”, 80 μl) was saved. Samples of chromatin were pre-cleared with M-280 sheep anti-rabbit IgG Dynabeads (Invitrogen) and immunoprecipitated with primary antibody (10 μg of anti-REST, anti-H3K9ac or anti-H3K4me2, Milipore). Immunocompelxes were collected on the Dynabeads and eluted with elution buffer (50 mM Tris, 10mM EDTA, 1% SDS). Following elution, samples were treated with proteinase K and purified by means of a PCR purification kit (Qiagen). For technical validation experiments, ChIP output of anti-REST-precipitated chromatin and total chromatin were

subjected to whole-genome amplification and then used for qPCR. miR-9, miR-124a, and miR-132 promoter abundance was quantified by real-time PCR using miR-specific primers ; miR-132 PR1 (F; CACCTCCAGAGCAGGCAAA, R; GGAGGCTGTGGCTCTATAAGGA), miR-132 PR2 (F; AAAGGGTCCTTAACACAGCAAAAA, R; ACAATGCATTAGAGACCAGGTGAA), miR-132 PR3 (F; TTGCGATGTTATGGGACCAA, R; GGGAGTACACGGAGGAGAAACTT), miR-9-1 (F; CCCCCAGCAATTTTCACATC, R; GGAGCCGGTTTTGTGCAA), miR-9-2 (F; GCGTCTTGCTGGCGTACTG, R; TGATGGCCCTTTTCTCCTTCT), miR-9-3 (F; CTGGCGAGACCTCGACCTT, R; CCGCAGACCCCCAGAGT), miR-124a-1 (F; CCTCCCCTTTGCAGGAAAAA, R; CCTCCGTAGGCTCTTTGTTCTC), miR-124a-2 (F; TCGGCCTGGGATTCTGATC, R; CTTTGATAGCCCGGTTTTGATT), miR-124a-3 (F; TGGAGTGGCGTTTTTTGGA, R; ACCTTGATCTGGGCCTTTAGG).

qRT–PCR

For cultures, neurons were washed twice with ice-cold PBS, and for tissue samples, the CA1 was rapidly microdissected. RNA was extracted using mirVana (Ambion), and concentration was measured with a Nanodrop (NanoDrop Technologies). Aliquots of RNA (10 ng) were reverse-transcribed to cDNA with the TaqMan®MicroRNA Reverse Transcription Kit (Appiled Biosystems). Quantitative real-time PCR (qPCR) was performed using TaqMan probes (Applied Biosystems) for miR-132 (Ref: 000457); miR-9 (Ref: 000583); miR-124a (Ref: 001182); and U6 snRNA (Ref: 001973) served as an endogenous reference. Reactions were run in triplicate in a StepOnePlus real-time PCR system (Applied Biosystems). The relative change in the miRNA expression was determined by the equation: Fold change = $2^{-\Delta Ct}$, ΔCt = Ct target – Ct reference) [58]. The Ct value corresponds to the cycle number at which the fluorescence signal crosses a threshold. Relative expression ratios were calculated by normalization of values for experimental samples to those of the corresponding control neurons or sham controls.

miR-132 overexpression constructs and lentiviral vectors

miR-132 overexpression constructs were kind gifts from Dr. Edbauer. For miR-132 expression in cultured hippocampal neurons and in the hippocampal CA1 in vivo, the oligonucleotides for miR-132 (sense; GATCGTCGACCCTGAAAGCCCCGCCCCCGCGTCTCCAGGGCAACCGTGGCTTTC GATTGTTACTGTGGGAACCGGAGGTAAC, antisense; GATCGTCGACGAGTGGTGGGGAGCGTGGGCGTGCTGCGGGGCGACCATGGCTG TAGACTGTTACCTCCGGTTCCCACAG) as described [16]. or the negative control miR (Invitrogen, sense; GATCGTCGACCCTGAAAGCCCCGCCCCCGCGTCTCCAGGGCAAAAATGTACTGC GCGTGGAGACGTGGGAACCGGAGTCTCC, anti-sense; ACGTGGGAACCGGAGTCTCCACGCAGTACATTTTCGCCCCGCAGCACGCCCACG CTCCCCACCACTCGTCGACGATC) were annealed, extended by Klenow fragment and cloned into a self-inactivating lentiviral vector pRRLsin.cPPT.CMV.eGFP.Wpre. High-titer vesicular stomatitis virus-pseudo-typed lentiviral stocks were produced in HEK-293T cells as described [9,59]. In brief, cells were transfected by means of calcium phosphate with a

pRRL.PPT.hCMV.GFP.shRNA.Wpre transfer construct, pMDLg/pRRE and pRSV-REV packaging constructs, and the envelope protein construct pMD2.G. Titers were calculated by infecting HEK-293T cells with serial dilutions of concentrated lentivirus, and eGFP fluorescence was evaluated by flow cytometry at 48 h after transduction; Titers were 1×10^9 transducing units. The expression of miR-132 was examined by qPCR 7 d following transduction of primary hippocampal neurons prepared from E18 Sprague Dawley rats.

Injection of lentiviral miR-132 in vivo

Lentiviral miR-132 or negative control miR was administered to rats by stereotaxic injection 14 d prior to global ischemia or sham surgery. A total volume of 4 μl of viral solution was injected directly into the right CA1 (3.0 mm posterior and 2.0 mm lateral to bregma, 4.0 mm below the skull surface) using a 28-gauge needle and a Hamilton syringe at a flow rate of 0.5 μl/min.

Histology and Fluoro-Jade labeling

Neuronal cell loss was assessed by histological examination of the dorsal hippocampus from animals killed at 7 d after sham surgery or global ischemia. Animals were anesthetized with halothane and transcardially perfused using 0.9% saline with heparin followed by ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.4. Brains were removed and immersed in fixative. Coronal sections (20 μm) were cut at the level of the dorsal hippocampus with a cryotome and processed for staining with toluidine blue or Fluoro-Jade C (Millipore).

Statistical analysis

For qRT-PCR, significance was assessed by means of the randomization test [60] and a Student's t-test (unpaired, two-tailed). For Western band densities and radioactive band densities, significance was assessed by means of the Student's t-test (unpaired, two-tailed). $P < 0.05$ was considered to be statistically significant.

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Highlights

- Global ischemia triggers a decrease in miR-132 expression in CA1 neurons destined to die.
- REST orchestrates epigenetic remodeling and silencing of miR-132 in insulted CA1 neurons.
- The gene silencing factor REST is critical to ischemia-induced loss of miR-132 in CA1.
- Loss of miR-132 is causally-related to neuronal death associated with global ischemia.
- Overexpression of miR-132 protects against ischemia-induced neuronal death.

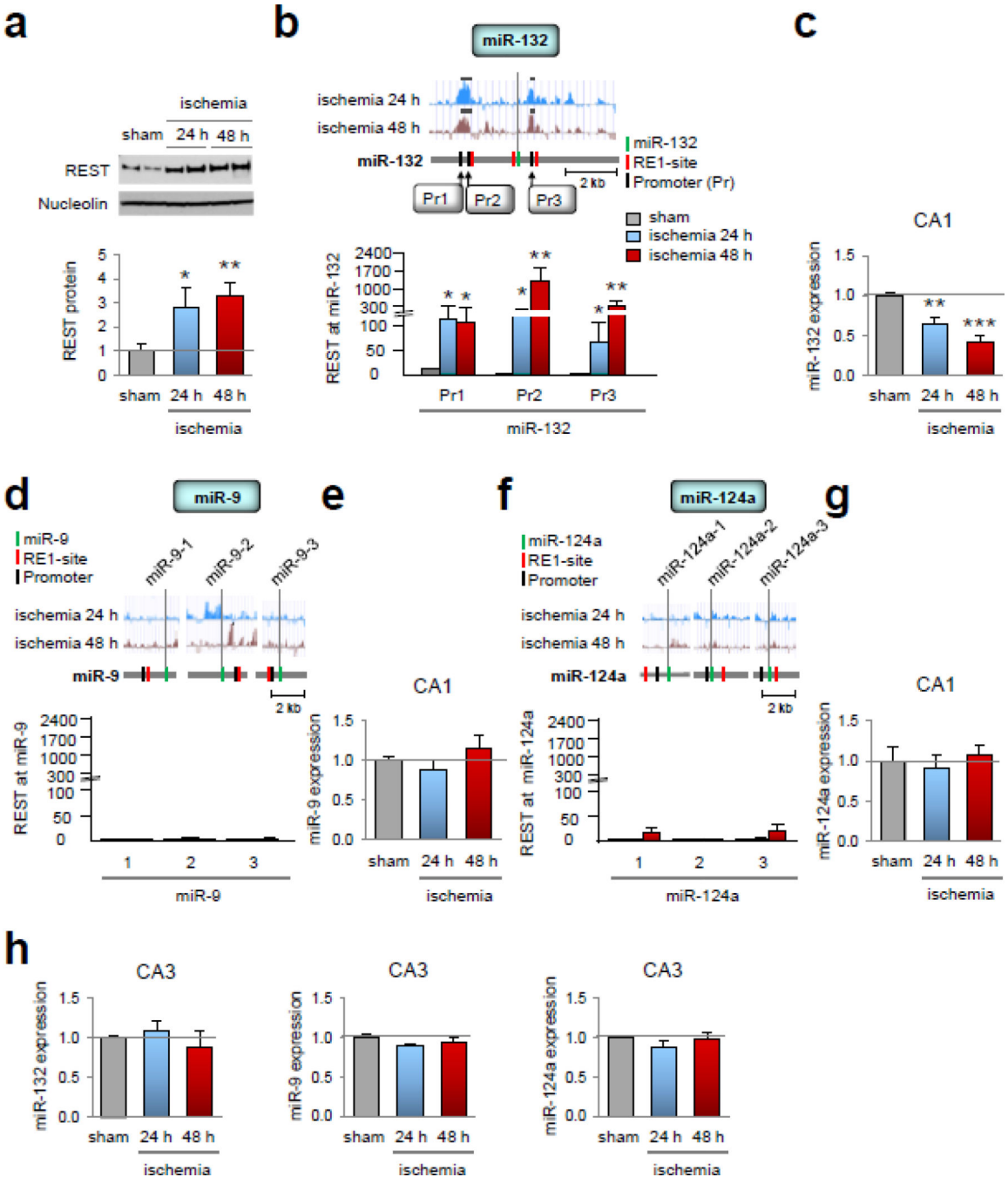

Figure 1. Global ischemia activates and recruits REST to the miR-132 (but not miR-9 or miR-124a) promoter, and silences miR-132 in CA1

(**a**) *Upper*, representative Western blot showing that ischemia increases REST in the CA1 at 24 h and 48 h after insult. *Lower*, summary data. (**b**) *Upper*, REST profile at the miR-132 promoter was assessed by ChIP-on-chip analysis in the postischemic CA1. *Lower*, single-locus ChIP, followed by qPCR, shows that REST is enriched at the miR-132 promoter (technical validation). (**c**) qRT-PCR shows that miR-132 expression is decreased (biological validation). (**d**) *Upper*, REST profile at the miR-9 promoter was assessed by ChIP-on-chip

analysis in the postischemic CA1. *Lower*, single-locus ChIP, followed by qPCR, shows that the miR-9 promoter, which contains an RE1 site, does not exhibit enhanced REST association (technical validation). (**e**) qRT-PCR shows that miR-9 expression is not altered (biological validation). (**f**) *Upper*, REST profile at the miR-124a promoter was assessed by ChIP-on-chip analysis in the postischemic CA1. *Lower*, single-locus ChIP-qPCR, shows that the miR-124a promoter, which contains an RE1 site, does not exhibit enhanced REST association (technical validation). (**g**) qRT-PCR shows that miR-124a expression is not altered (biological validation). (**h**) qRT-PCR shows that miR-132, miR-9 and miR-124a expression is not altered in postischemic CA3. $n = 3$ per treatment group/ 3 independent experiments. Data represent mean ± SEMs. ***, $p < 0.001$, **, $p < 0.01$, *,$p < 0.05$.

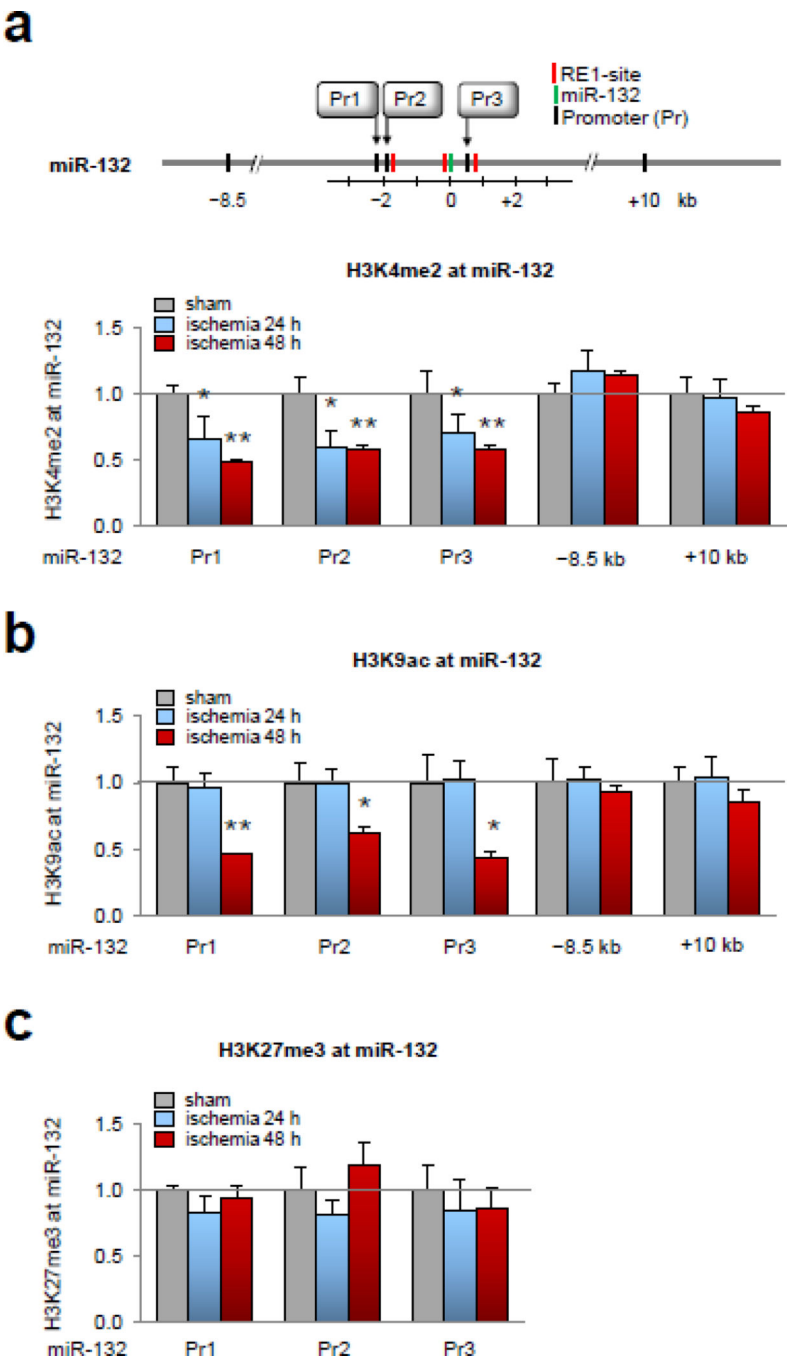

Figure 2. REST orchestrates epigenetic remodeling at the miR-132 promoter

(**a**) Single-locus ChIP-qPCR showing that ischemia induced a decrease in H3K4me2 at the miR-132 promoter, but not at sites 8.5 kb upstream or 10 kb downstream from miR-132 in the CA1, evident at 24 h and 48 h. (**b**) Single locus ChIP-qPCR showing that ischemia induced a decrease in H3K9ac at the miR-132 promoter in CA1 at 48 h, but not at sites 8.5 kb upstream or 10 kb downstream form miR-132. (**c**) Single locus ChIP-qPCR showing that ischemia did not alter trimethylation of H3K27 (H3K27me3) at the miR-132 promoters. *n* =

3 per treatment group/ 3 independent experiments. Data represent mean ± SEMs. **, $p < 0.01$, *,$p < 0.05$.

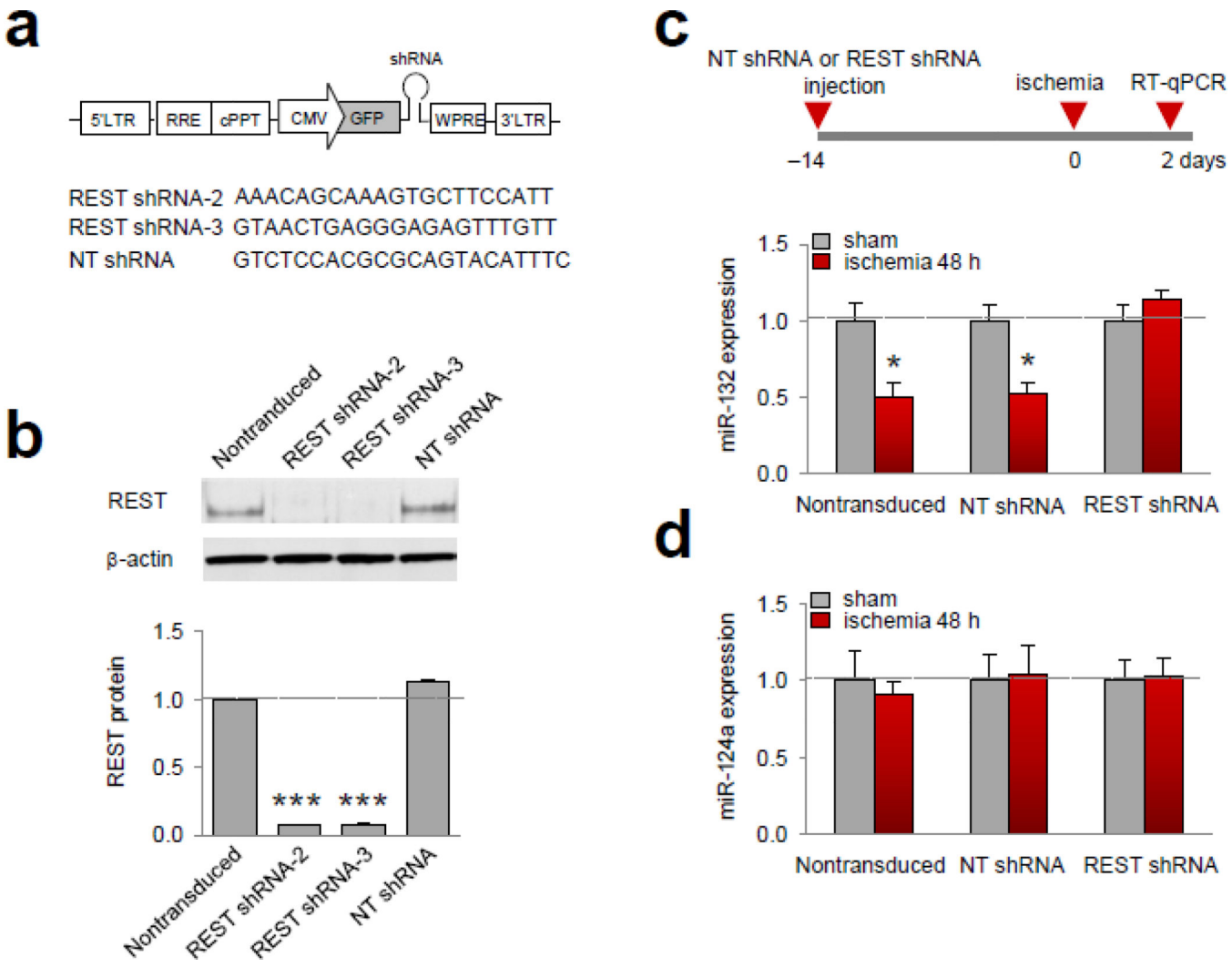

Figure 3. RNAi-mediated depletion of REST prevents the ischemia-induced decrease in miR-132

(**a**) REST shRNA and non-targeting (NT) shRNA were cloned into the lentivirus expression system. (**b**) *Upper*, representative Western blots showing shRNAs targeting two different sequences in REST (REST shRNA-2, REST shRNA-3) knockdown REST in hippocampal neurons. *Lower*, summary data. (**c**) *Upper*, time line. *Lower*, qRT-PCR showing that REST shRNA rescues the ischemia-induced decrease in miR-132 expression level in CA1 at 48 h. (**d**) qRT-PCR showing that either ischemia or REST shRNA does not alter miR-124a expression level. n = 3~4 per treatment group/ 3 independent experiments. *, $p < 0.05$.

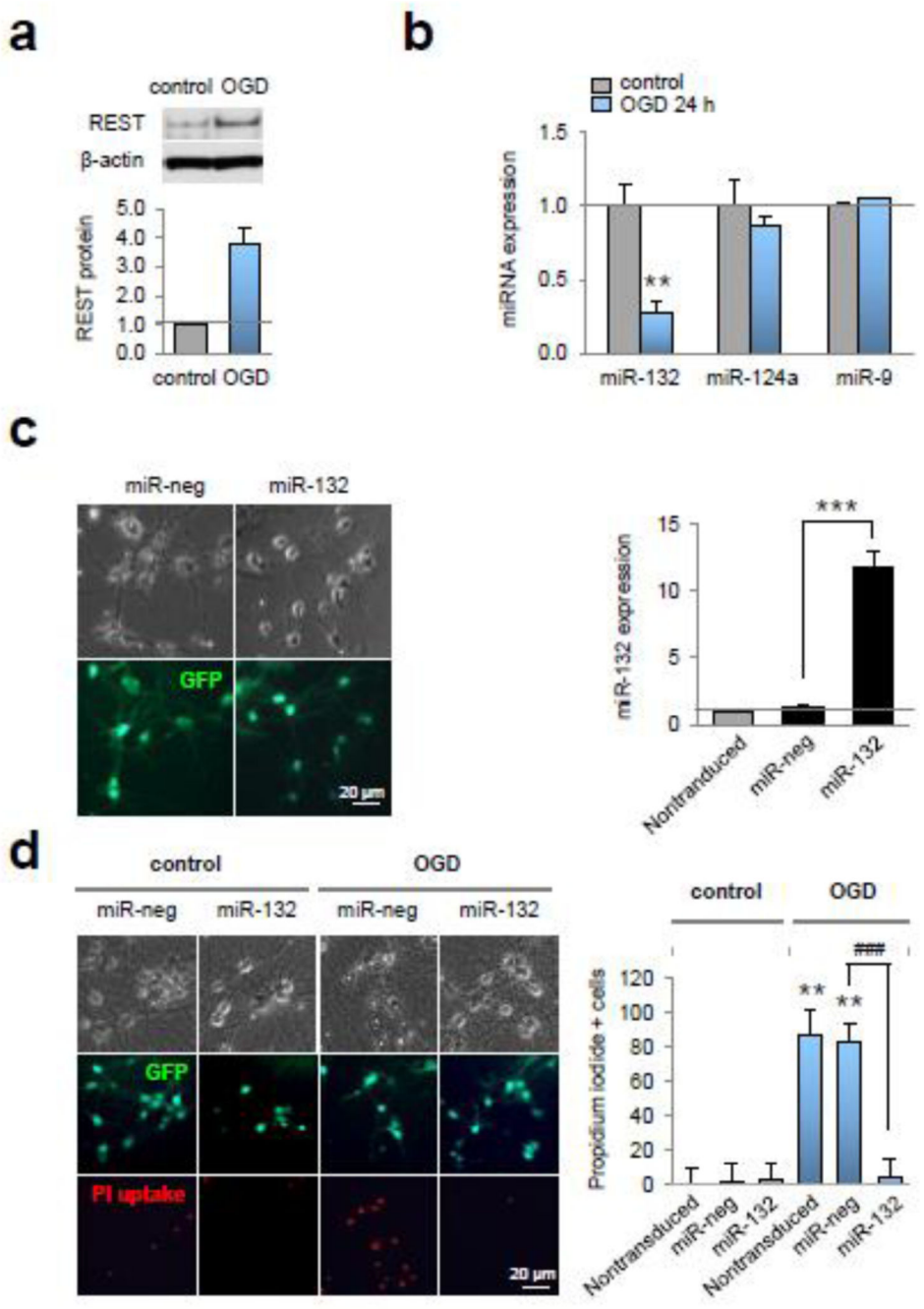

Figure 4. Overexpression of miR-132 enables CA1 neurons to survive

(**a**) *Upper*, representative Western blot showing that oxygen-glucose deprivation (OGD) increases REST in primary cultures of hippocampal neurons *in vitro*. *Lower*, summary data. (**b**) OGD decreases miR-132 expression in hippocampal neurons *in vitro*. (**c**) *Left*, representative GFP fluorescence images showing expression of miR-132 and negative control miR (miR-neg) in hippocampal neurons. *Right*, qRT-PCR showing that overexpression of miR-132 by means of the lentivirus expression system increases miR-132 levels in hippocampal neurons. (**d**) *Left*, representative images showing that overexpression

of miR-132, but not miR-neg, rescues hippocampal neurons from OGD-induced death, assessed by propidium iodide uptake. *Right*, summary data. $n = 3$ per treatment group/ 3 independent experiments. $^{###}$, $p < 0.001$, **, $p < 0.01$.

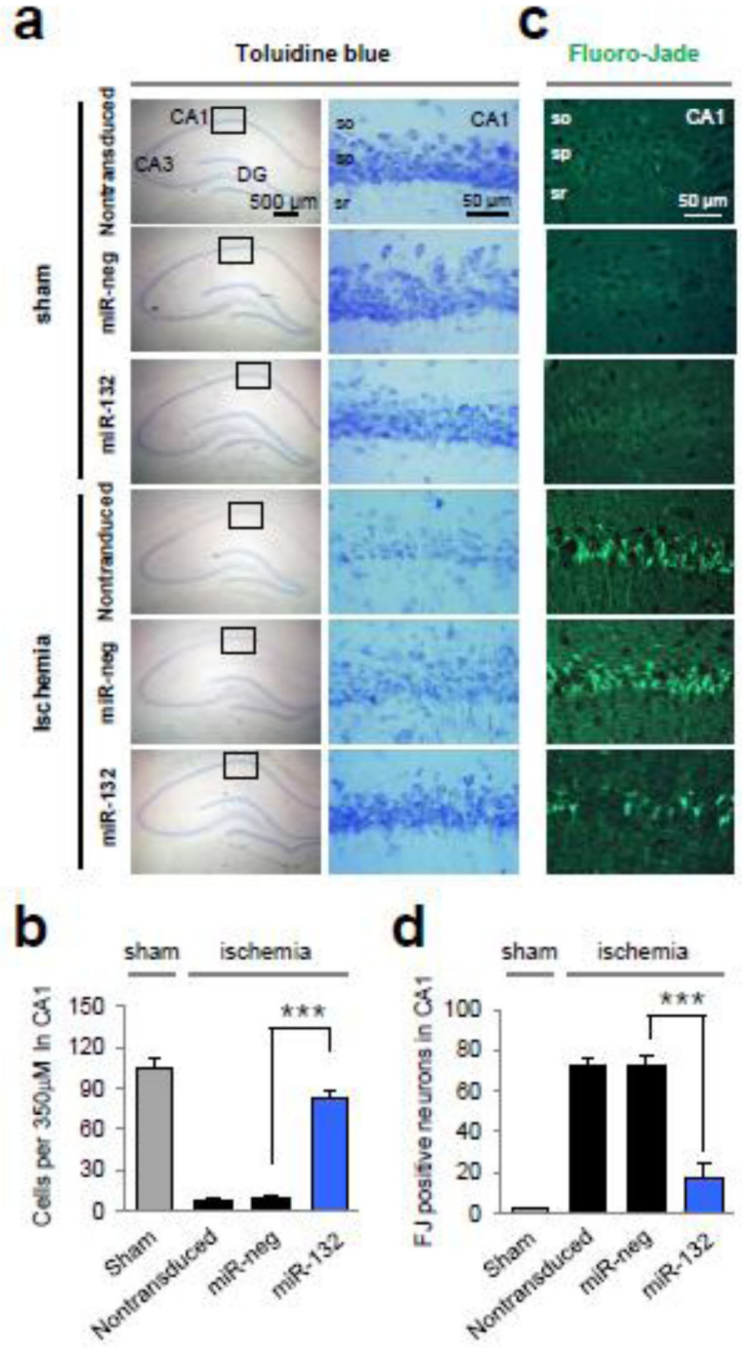

Figure 5. Overexpression of miR-132 protects CA1 neurons from ischemia-induced cell death *in vivo*

(**a**) Representative images of brain sections stained with toluidine blue from animals expressing miR-132 or miR-neg, and subjected to global ischemia or sham operation at 7 d after surgery. (**b**) Summary data showing the number of living CA1 pyramidal neurons, as assessed by toluidine blue. (**c**) Representative images of brain sections stained with Fluoro-Jade (**d**) Summary data showing the number of degenerating CA1 pyramidal neurons, as

assessed by Fluoro-Jade ($n = 4 - 6$ animals). ***, $p < 0.001$. so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum.