

Ischemic insults promote epigenetic reprogramming of μ opioid receptor expression in hippocampal neurons

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Transient global ischemia is a neuronal insult that induces delayed, selective death of hippocampal CA1 pyramidal neurons. A mechanism underlying ischemia-induced cell death is activation of the gene silencing transcription factor REST (repressor element-1 silencing transcription factor)/NRSF (neuron-restrictive silencing factor) and REST-dependent suppression of the AMPA receptor subunit GluR2 in CA1 neurons destined to die. Here we show that REST regulates an additional gene target, OPRM1 (μ opioid receptor 1 or MOR-1). MORs are abundantly expressed by basket cells and other inhibitory interneurons of CA1. Global ischemia induces a marked decrease in MOR-1 mRNA and protein expression that is specific to the selectively vulnerable area CA1, as assessed by quantitative real-time RT-PCR, Western blotting, and ChIP. We further show that OPRM1 gene silencing is REST-dependent and occurs via epigenetic modifications. Ischemia promotes deacetylation of core histone proteins H3 and H4 and dimethylation of histone H3 at lysine-9 (H3-K9) over the MOR-1 promoter, a signature of epigenetic gene silencing. Acute knockdown of MOR-1 gene expression by administration of antisense oligodeoxynucleotides to hippocampal slices *in vitro* or injection of the MOR antagonist naloxone to rats *in vivo* affords protection against ischemia-induced death of CA1 pyramidal neurons. These findings implicate MORs in ischemia-induced death of CA1 pyramidal neurons and document epigenetic remodeling of expression of OPRM1 in CA1 inhibitory interneurons.

epigenetic modification | gene silencing | ischemia | neuronal death

The transcriptional repressor REST silences a large network of genes essential for neuronal function including neurotransmitter receptors, ion channels, synaptic vesicle protein, and neuronal trophic factors (1–5) and restricts their expression to neurons in a variety of genetic contexts. During the late stages of neuronal differentiation, REST activation is important for the acquisition of the neural phenotype (5). Mice with targeted deletion of the REST gene die very early in development (6), and overexpression of REST in differentiating neurons disrupts neuronal gene expression and increases axon-guidance error (4).

REST is a Krüppel-type zinc-finger transcription factor that interacts with a 21-bp cis-element RE1 (repressor element 1, also called NRSE, neuron-restrictive silencing element) found in the regulatory regions of neuron-specific target genes (1, 2, 7, 8). REST silences target genes via association with distinct corepressor platforms involving mSin3A (9–11) and CoREST (5, 12), which recruit histone deacetylases (HDACs) 1 and 2 (5, 9, 10, 13–17). HDACs silence gene transcription by deacetylation of core histones and tightening of the core chromatin complex (18, 19). In addition, CoREST effects long-term gene silencing by association with methyl-CpG binding protein 2 (MeCP2), site-specific histone methyltransferase G9a (13, 20–22), which promotes dimethylation of histone 3 at lysine-9 (H3-K9) (23, 24), and the newly discovered histone demethylase LSD1, which removes methyl groups from lysine-4 of histone 3 (H3-K4) (25, 26). Whereas dimethylation of histone H3-K9 is an epigenetic

marker of gene silencing, trimethylation of H3-K4 is a marker of active transcription (18, 19).

MORs are members of the large superfamily of G protein-coupled receptors and are activated by endogenous opioid peptides (27, 28). Upon activation, MORs inhibit adenylyl cyclase, reduce Ca^{2+} currents, stimulate inwardly rectifying K^{+} currents and activate mitogen-activated protein kinases via pertussis toxin-sensitive Gi/G0 proteins. Pharmacological studies indicate the existence of three MOR subtypes: μ_1 , μ_2 , and morphine-6 β -glucuronide receptors (27, 28). Studies involving mice with targeted deletion of the OPRM1 gene implicate MORs in the euphoria and craving of exogenous opiates and other drugs of abuse (29). Within CA1, MORs are abundantly expressed on the terminals of basket cells, GABAergic inhibitory interneurons in the stratum pyramidale and strategically poised to influence synaptic efficacy in response to neuronal activity and/or insult (30–32). Upon activation, MORs inhibit release of GABA onto CA1 pyramidal cells, thereby increasing neuronal excitability (33) and epileptogenesis (34).

The present study was undertaken to determine the impact of global ischemia on MOR expression in CA1 and to examine a possible role for MORs in ischemia-induced death of CA1 pyramidal neurons. Here we show that REST binds and suppresses MOR promoter activity and gene expression specifically in the CA1 subfield where pyramidal cells die; presumably the suppression is restricted to the inhibitory interneurons, which actually survive the ischemic insult. Acute knockdown of MOR expression by application of antisense oligodeoxynucleotides (ODN) *in vitro* or pharmacological blockade of MORs by injection of the antagonist naloxone *in vivo* protects hippocampal neurons against ischemic damage. These observations implicate MORs in the selective, delayed death of CA1 neurons and identify MORs as a target for therapeutic intervention and amelioration of neuronal cell loss and cognitive deficits associated with global ischemia.

Results

Global Ischemia Suppresses MOR-1 mRNA and Protein in Vulnerable CA1 Neurons. The role of MORs in ischemia-induced neuronal death is not well delineated. To address this issue, we subjected rats to global ischemia or sham operation. Transient global or

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The authors declare no conflict of interest.

Abbreviations: ODN, oligodeoxynucleotide; HDAC, histone deacetylase; MOR, μ opioid receptor; PI, propidium iodide; OGD, oxygen/glucose deprivation.

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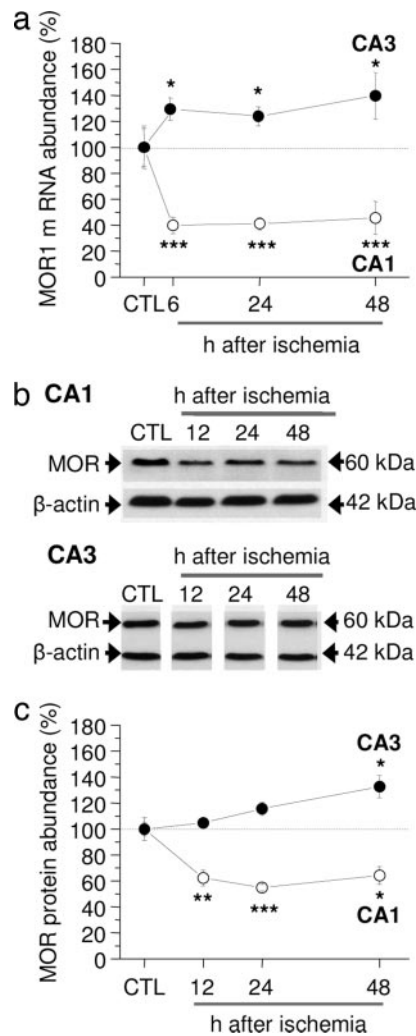


Fig. 1. Global ischemia decreases MOR-1 mRNA and protein expression in CA1. (a) Real-time PCR of RNA samples from the CA1 and CA3 of control and experimental animals at 6, 24, and 48 h after ischemia. Bars represent relative mRNA quantity measured by the Ct values \pm SEMs, where Ct is the number of cycles needed for detection of the fluorescence signal; significance was assessed by using the REST software with which transcript differences are tested for significance by a pairwise fixed reallocation–randomization test (55). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (b and c) Representative Western blots probed with anti-MOR antibody (b) and relative MOR abundance in samples from the CA1 and CA3 at 48 h after sham operation (control) or at 12, 24, and 48 h after ischemia (c). Values are expressed as the ratio of band densities for experimental samples to band density of the corresponding control sample after correction for loading. Bars represent mean \pm SEMs. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (Student's unpaired t test).

forebrain ischemia in rats provides a well established model of neuronal insult in which there is a highly specific, delayed death of CA1 pyramidal neurons (35). Because ischemia activates the transcriptional repressor REST specifically in CA1 (14, 36), and MOR-1 is a known REST target (37, 38), we assessed the time course of MOR-1 mRNA expression in CA1 and CA3 by quantitative real-time PCR. In control CA1, MOR-1 mRNA expression was high ($n = 15$). Ischemia triggered a marked decrease in MOR-1 mRNA selectively in CA1 (decrease by: 6 h, $60 \pm 6\%$, $n = 6$, $P < 0.001$; 24 h, $58 \pm 4.8\%$, $n = 5$, $P < 0.001$; 48 h, $54 \pm 13\%$, $n = 5$, $P < 0.001$ vs. control) (Fig. 1a). In contrast, MOR-1 mRNA was enhanced in CA3 (increase by: 6 h, $29 \pm 8\%$, $n = 4$, $P < 0.05$; 24 h, $24 \pm 7\%$, $n = 4$, $P < 0.05$; 48 h, $39 \pm 18\%$, $n = 4$, $P < 0.05$) (Fig. 1a).

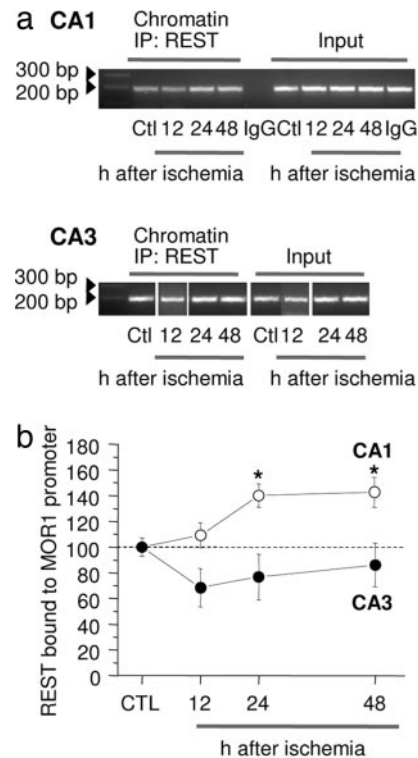
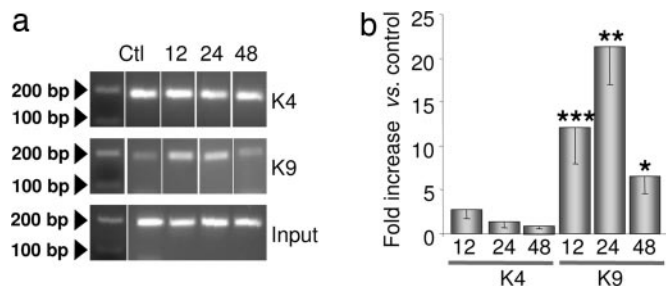


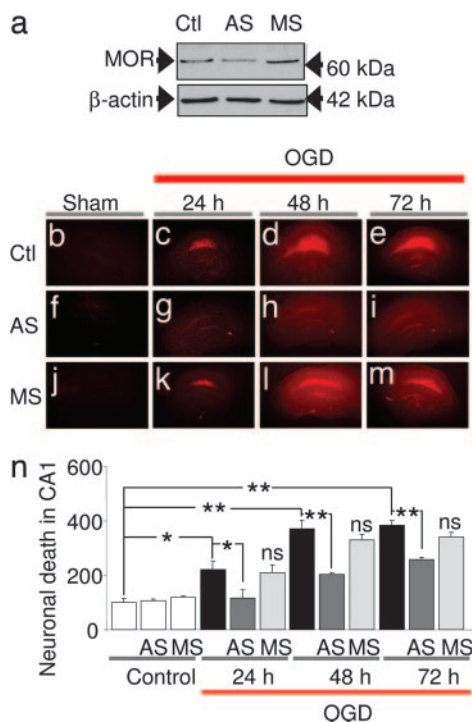
Fig. 2. Ischemia enhances REST binding to the MOR-1 promoter in CA1. (a) ChIP analysis of cross-linked chromatin from the CA1 and CA3 of control (Ctl) and experimental animals at 12, 24, and 48 h after ischemia immunoprecipitated with an anti-REST antibody. Immunoprecipitation with nonimmune IgG showed negligible PCR product, indicating specificity of the ChIP technique. Left lanes show ODN length standards. (b) Band densities for PCR products were normalized to that of the corresponding control input. Bars represent mean \pm SEMs. *, $P < 0.05$ (Student's unpaired t test).

Because mRNA changes are not always followed by changes in protein abundance, we next examined the impact of ischemic insults on MOR protein in CA1 and CA3 by Western blot analysis. In control hippocampus, MOR protein expression, evident as a band of $M_r = 60$ kDa, was robust (Fig. 1b). Ischemia markedly decreased MOR protein abundance in CA1, evident as late as 48 h (decrease by: 12 h, $38 \pm 6\%$, $n = 7$, $P < 0.005$ vs. control; 24 h, $46 \pm 2\%$, $n = 7$, $P < 0.0005$; 48 h, $36 \pm 7\%$, $n = 5$, $P < 0.05$ vs. control; shams, $n = 8$) (Fig. 1c). In contrast, ischemia did not significantly alter MOR protein abundance in CA3 at 12 h and 24 h and modestly increased MOR protein at 48 h (increase by $32 \pm 8\%$, $n = 5$, $P < 0.05$) (Fig. 1c). Thus, ischemia-induced changes in MOR-1 mRNA and MOR protein are cell-specific and are consistent with the possibility that REST silences MOR-1 expression.

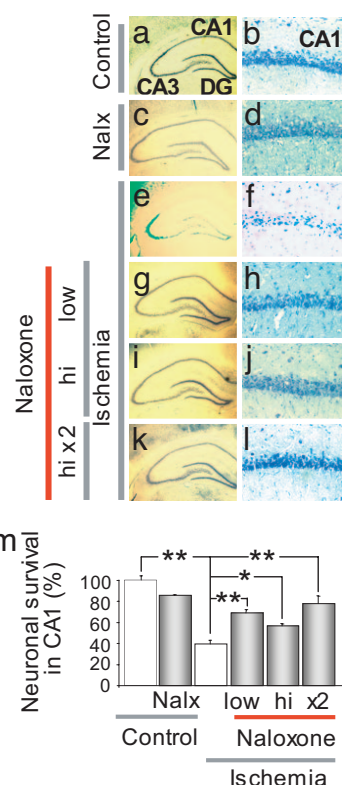
Ischemia Promotes Association of REST with the MOR-1 Promoter in CA1. The finding, that global ischemia silences MOR-1 mRNA and protein expression in selectively vulnerable CA1 neurons, prompted us to analyze chromatin for association of REST with the promoter of the MOR-1 gene by ChIP assay. In control CA1, REST was bound to the RE1 region of the MOR-1 promoter at low, but significant levels (Fig. 2a, Ctl lanes). Ischemia enhanced association of REST with the MOR-1 promoter in the CA1, but not CA3, of experimental animals, evident at 24 and 48 h after ischemia (increase by: 24 h, $40 \pm 9\%$, $n = 4$, $P < 0.05$ vs. control; 48 h, $42 \pm 11\%$, $n = 4$, $P < 0.05$ vs. control) (Fig. 2a, center lanes). In contrast, ischemia did not significantly alter REST over the MOR-1 promoter in CA3 at times examined (Fig. 2b, center lanes). REST immunoreactivity was negligible in the



down-regulation of MOR protein, as assessed by Western analysis of whole slices (Fig. 5a). In contrast, missense did not significantly alter MOR protein (Fig. 5a). OGD (30 min) induced selective, delayed death of CA1



pyramidal neurons, evident at 24 h and maximal at 72 h, as assessed by PI uptake ($n = 8$ per time point) (Fig. 5 c-e and n). MOR-1 antisense suppressed OGD-induced neuronal death at 24 h (reduction by $46 \pm 9\%$, $n = 5$, $P < 0.01$ vs. OGD), 48 h (reduction by $62 \pm 9\%$, $n = 6$, $P < 0.05$ vs. OGD), and 72 h (reduction by $63 \pm 9\%$, $n = 8$, $P < 0.01$ vs. OGD) (Fig. 5 g-i and n). In contrast, MOR-1 missense ($n = 4$) (Fig. 5 k-n) did not detectably alter neuronal survival in slices subjected to OGD. Moreover, antisense and missense ODNs did not detectably alter cell survival in control slices subjected to normoxic conditions ($n = 6$ per treatment) (Fig. 5 f, j, and n). These findings demonstrate that MOR-1 antisense affords neuroprotection by suppressing MOR expression in CA1 and implicate MOR in ischemia-induced neuronal death.



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Naloxone Protects Against Ischemia-Induced Death of CA1 Neurons in Vivo. To examine whether MOR activity is causally related to ischemia-induced cell death in a clinically relevant animal model, we examined whether naloxone, a μ opioid antagonist, protects CA1 neurons when administered close to the time of ischemia. The role of opioid receptors in neuronal survival/cell death is not well delineated. To address this issue directly, we administered naloxone before or before and after ischemia. Ischemia induced extensive death of pyramidal cells in the CA1, assessed at 7 days after ischemia (reduction to $40 \pm 3.6\%$ of control, $n = 12$, $P < 0.01$ vs. control) (Fig. 6 e and f; summary data in Fig. 6m); the

few remaining pyramidal neurons were severely damaged and appeared pyknotic (Fig. 6, compare *e* and *f* with *a* and *b*). Neuronal death was specific in that little or no cell loss occurred in the nearby CA2 or transition zone, CA3 or dentate gyrus (Fig. 6*e* and not illustrated). Administration of naloxone directly into the right cerebral ventricle at 1 h before ischemia at 0.1 mg/kg (low dose) or 1.0 mg/kg (high dose) or at 1 h before and 1 h after ischemia at 1 mg/kg did not significantly alter neuronal survival in the hippocampus of control (sham-operated) animals (Fig. 6*c* and *d*) but afforded robust protection of CA1 neurons in animals subjected to global ischemia. The lower dose given before ischemia increased the number of surviving neurons to $69 \pm 3\%$ of control ($n = 6$, $P < 0.01$ vs. ischemia) (Fig. 6*g*, *h*, and *m*); the higher dose given before ischemia increased the number of surviving neurons to $56 \pm 2\%$ of control ($n = 5$, $P < 0.05$ vs. ischemia) (Fig. 6*i*, *j*, and *m*); the higher dose given before and after ischemia increased the number of surviving neurons to $74 \pm 7\%$ ($n = 5$, $P < 0.01$ vs. ischemia) (Fig. 6*k* and *l*; summary data in Fig. 6*m*, no significant difference between doses). These data demonstrate that block of MOR receptors by the competitive antagonist naloxone affords protection of CA1 neurons against ischemia-induced neuronal death and are consistent with a role for MOR silencing in neuronal survival.

Discussion

Global ischemia is a neuronal insult that induces delayed, selective death of CA1 pyramidal neurons. A mechanism underlying ischemia-induced cell death is activation of the transcriptional repressor REST and REST-dependent silencing of the AMPAR subunit GluR2 in neurons destined to die (14). Here we show that global ischemia silences an additional REST target, MOR-1. Ischemia induces a marked down-regulation in MOR-1 mRNA and protein expression in selectively vulnerable CA1 (but not CA3). These findings document REST induction and silencing of MOR-1 expression in postischemic CA1, presumably in inhibitory interneurons. Ischemia promotes association of REST with the MOR-1 promoter in CA1 (but not CA3), consistent with a causal relation between REST induction and MOR-1 silencing in CA1. We further show that ischemia promotes deacetylation of histones H3 and H4 and dimethylation of H3-K9 (but not H3-K4) over the MOR-1 promoter in CA1 neurons destined to die, a signature of epigenetic gene silencing. To our knowledge, these findings document the first example of insult-induced histone methylation in neurons and implicate histone modification in the pathogenesis of neurodegeneration after global ischemia. We further show that MOR-1 expression is causally related to ischemia-induced neuronal death. Acute knockdown of MOR-1 gene expression by administration of antisense ODNs to hippocampal slices *in vitro* or intracerebroventricular injection of the MOR antagonist naloxone to rats *in vivo* affords protection against ischemia-induced death of CA1 neurons. These observations identify MORs as a novel target for therapeutic intervention and amelioration of the deleterious consequences associated with global ischemia. They also extend observations that the MOR agonist fentanyl exacerbates neurodegeneration after global ischemia (44).

Epigenetic Remodeling of Insulted CA1 Neurons. Posttranslational modification of histone amino-terminal tails is an important mechanism that dynamically regulates chromatin structure and gene transcription. A fundamental mechanism by which REST silences target genes is by recruitment of the corepressors mSin3A and CoREST, which in turn recruit HDACs to the promoters of target genes (13, 21, 22). HDACs are implicated in dynamic gene repression and silence target genes by removal of acetyl groups from the N-terminal tails of histone proteins and tightening of the core chromatin complex. Our finding that ischemia promotes REST-dependent deacetylation of core histones H3 and H4 over the MOR-1 promoter is consistent with dynamic, REST-dependent

gene silencing. The REST/CoREST complex additionally contains the site-specific histone methyltransferase G9a (20). Our finding that ischemia promotes dimethylation of H3-K9 (but not H3-K4) over the MOR-1 promoter is consistent with long-term, REST-dependent gene silencing via G9a-dependent histone methylation. Together, the results document epigenetic remodeling of MOR-1 gene expression in insulted CA1 neurons.

MOR-1 and Neuronal Survival. Our findings that MOR-1 gene silencing and pharmacological blockade promote cell survival are consistent with findings of others that pharmacological blockade of MOR is protective in *in vivo* and *in vitro* models of hypoxia-ischemia (45–48) and that mice with targeted deletion of exon-1 of the MOR-1 gene exhibit enhanced survival of dentate gyrus neurons (41) in development. Whereas the protective effects of naloxone have been ascribed not to its binding to classical opioid receptors, but rather to its anti-inflammatory actions, the observation that (–) naloxone, but not its inert stereoisomer (+) naloxone, is protective indicates a direct opioid receptor-mediated action (47). Our finding that MOR-1 silencing or blockade affords neuroprotection is consistent with findings that MOR blockade reduces the abundance of Fas, FasL and proapoptotic BCL-2 family members (Bad and Bax) in the mouse neocortex (49). Under physiological conditions, activation of MOR inhibits GABA release from inhibitory interneurons that selectively project onto the somata of pyramidal neurons, thereby enhancing the excitability and output of pyramidal neurons (50). A possible scenario is that ischemia-induced gene silencing of MOR disinhibits GABA release from inhibitory interneurons and attenuates excitability and activity of CA1 pyramidal neurons and reduces excitotoxicity of endogenous glutamate (51). In this light, MOR-1 silencing would represent a failed attempt of postischemic inhibitory interneurons to promote survival of CA1 pyramidal neurons.

Significance of Epigenetic Remodeling of Insulted Neurons. Our findings that ischemia triggers REST-dependent histone modifications and gene silencing of MOR-1 (the present study) and AMPA receptor GluR2 gene expression (14) implicate REST as a compelling candidate for orchestrating epigenetic modifications in postischemic neurons. Our findings that ischemia triggers REST expression in neurons destined to die (ref. 14 and the present study) and that REST expression is causally related to neuronal death (14) implicate REST-activated epigenetic modifications and gene silencing in neuronal injury. The topic of REST-dependent epigenetic modifications and reprogramming of mature neurons is a new and emerging area of investigation. Dysregulation of REST and its target genes is implicated in pathogenesis of Down's syndrome (55), Alzheimer's disease (56), and status epilepticus (14, 36, 57). The present study adds neurodegeneration after global ischemia to the growing list of neurological disorders and diseases in which REST-mediated epigenetic modifications and gene silencing are dysregulated.

Materials and Methods

Global Ischemia and Naloxone Administration. Male Sprague–Dawley rats (150–200 g) were subjected to transient global ischemia or sham operation by four-vessel occlusion as described (14, 36). At indicated times, animals were anesthetized and injected intracerebroventricularly with naloxone or saline [for details, see supporting information (SI) *Materials and Methods*].

Real-Time PCR. To assess MOR-1 mRNA abundance, animals were killed at times after surgery and CA1 and CA3 were removed. RNA isolation, reverse transcription, and DNA amplification were performed according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). Primers are as described in *SI Materials and Methods*.

Western Blot Analysis. Protein extracts were prepared from CA1 and CA3 of control and experimental animals and subjected to Western blot analysis (14). Blots were probed with an anti-MOR antibody (*SI Materials and Methods*).

ChIP Assays. ChIP assays were performed according to the protocol from an Upstate Biotechnology ChIP kit as described (14). The following antibodies were used: anti-REST, anti-acetylated H3, anti-acetylated H4, anti-methylated H3 raised against dimethyl K4 H3, anti-methylated H3 raised against dimethyl K9 H3, and non-immune rabbit IgG (*SI Materials and Methods*).

Slice Cultures and Antisense ODN Administration. Hippocampal slice cultures were prepared and subjected to OGD as described (14, 36). For probes, see *SI Materials and Methods*.

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