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DNA Methyltransferase Inhibitors Coordinately Induce Expression of the Human Reelin and Glutamic Acid Decarboxylase 67 Genes

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ABSTRACT

Reelin and glutamic acid decarboxylase 67 (GAD67) mRNAs and protein levels are substantially reduced in postmortem brains of patients with schizophrenia. Increasing evidence suggests that the observed down-regulation of reelin and GAD67 gene expression may be caused by dysfunction of the epigenetic regulatory mechanisms operative in cortical GABAergic interneurons. To explore whether human reelin and GAD67 mRNAs are coordinately regulated through DNA methylation-dependent mechanisms, we studied the effects of DNA methyltransferase inhibitors on reelin and GAD67 expression in NT-2 neuronal precursor cells. Competitive reverse transcription-polymerase chain reaction with internal standards was used to quantitate mRNA levels. The data showed that reelin and GAD67 mRNAs are induced in the same dose- and time-dependent manners. We further demonstrated that the activation of these two genes correlated with a reduction in DNA methyltransferase activity and DNA methyltransferase 1 (DNMT1) protein levels. Time course Western blot analysis showed that DNMT1 protein down-regulation occurs temporally before the reelin and GAD67 mRNA increase. In addition, chromatin immunoprecipitation assays demonstrated that the activation of the reelin gene correlates with the dissociation of DNMT1 and methyl-CpG binding protein 2 (MeCP2) from the promoter, and an increased acetylation of histones H3 in the region. Together, our data strongly imply that human reelin and GAD67 genes are coordinately regulated through epigenetic mechanisms that include the action of DNMT1. Our study also suggests that negative regulation of the reelin gene involves methylation-dependent recruitment of DNMT1, MeCP2, and certain histone deacetylases, which most likely reduce the activity of the promoter by shifting the surrounding chromatin into a more compact state.

It is now well established that disruption of epigenetic mechanisms can give rise to a variety of disorders in humans, including some that are associated with cognitive abnormalities (Egger et al., 2004; Jiang et al., 2004; Levenson and Sweatt, 2005). For instance, mutations in the methyl-CpG binding protein MeCP2 are responsible for 90 to 95% of all cases of Rett syndrome, one of the most common causes of mental retardation in the female population (Weaving et al., 2005). These findings, together with studies using conditional DNA methyltransferase 1 (DNMT1) mutant mice, suggest that DNA methylation is essential for proper neuronal function (Fan et al., 2001; Tucker, 2001). It seems that DNA methylation may be an important mechanism associated with the dynamic regulation of genes expressed in neurons, especially those involved in synaptic plasticity, such as reelin and brain-derived neurotrophic factor (Martinowich et al., 2003; Levenson et al., 2006).

Increasing evidence indicates that the dysfunctions seen in schizophrenia may be caused by an epigenetically induced down-regulation of GABAergic neuronal markers, such as reelin and glutamic acid decarboxylase 67 (GAD67) (Costa et al., 2004; Guidotti et al., 2005). In adult brain, reelin most likely plays an important role in synaptic plasticity, learning, and memory formation (Qiu et al., 2006). GAD67 is one of the two key enzymes involved in the synthesis of GABA (Guidotti et al., 2005). The decreases in reelin and GAD67 mRNA and protein levels are among the most consistently replicated findings reported in postmortem brains of patients with schizophrenia (Fatemi et al., 2000; Guidotti et al., 2000; Eastwood and Harrison, 2003; Torrey et al., 2005). A recent study demonstrated that the same GABAergic neurons that

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ABBREVIATIONS: DNMT, DNA methyltransferase; AZA, 5-aza-2′-deoxycytidine; ChIP, chromatin immunoprecipitation; DOXO, doxorubicin; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; GAD67, glutamic acid decarboxylase 67; HDAC, histone deacetylase; MeCP2, methyl-CpG binding protein 2; NT-2, N-tera 2 neuronal progenitor cells; ZEB, zebularine; ANOVA, analysis of variance; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline.

express reelin and GAD67 exhibit an up-regulation of the mRNA that encodes DNMT1 (Veldic et al., 2004). We and others have also shown that the reelin promoter is hypermethylated in the brains of patients with schizophrenia compared with control subjects (Abdolmaleky et al., 2005; Grayson et al., 2005). Together, these findings support our hypothesis that down-regulation of reelin, GAD67, and probably other mRNAs and proteins expressed in GABAergic neurons may be caused by mechanisms mediated through DNMT1-induced hypermethylation of the corresponding CpG island-containing promoters (Grayson et al., 2006).

We have already accumulated evidence showing that the human reelin gene is epigenetically regulated through changes in the methylation status of the promoter. Using NT-2 neuronal precursor cells, we have shown that the reelin promoter is more heavily methylated when the gene is silent (Chen et al., 2002). Activation of the reelin gene by various agents, including retinoic acid, the DNA methylation inhibitor 5-aza-2′-deoxycytidine (AZA), and histone deacetylase (HDAC) inhibitors valproic acid and trichostatin A, corresponds with a decrease in promoter methylation. In addition, induction of reelin expression is accompanied by alterations that suggest a more open chromatin structure. These changes include the appearance of DNase I hypersensitive sites and increased levels of acetyl histone H3 and acetyl histone H4 histones in the vicinity of the reelin promoter (Chen et al., 2002; Mitchell et al., 2005).

Studies in mice indicate that reelin and GAD67 RNAs may be coordinately regulated. Treatment with L-methionine, a precursor of the methyl donor *S*-adenosyl-methionine (SAM), induced the down-regulation of reelin and GAD67 mRNAs and proteins in vivo (Tremolizzo et al., 2002) and in primary neuronal cell cultures in vitro (Noh et al., 2005). This effect of methionine was attenuated by cotransfection of DNMT1 antisense oligonucleotides, providing a link between the expression of DNMT1 and the regulation of reelin and GAD67 genes (Noh et al., 2005). In addition, methionine treatment also induced an increased association of the methyl CpG-binding protein MeCP2 to mouse reelin and GAD67 promoters (Dong et al., 2005).

The aim of the current study was to evaluate the hypothesis that the human reelin and GAD67 genes are coordinately regulated by DNA methylation through the action of DNMT1. To address this, we used neuronal precursor cells (NT-2) and treatments with three distinct DNA methyltransferase inhibitors. Doxorubicin (DOXO) has recently been shown to act as a potent inhibitor of DNMT1 activity, most likely acting through DNA intercalation (Yokochi and Robertson, 2004). AZA and zebularine (ZEB) are nucleoside analogs that after incorporation into replicating DNA form covalent bonds with DNA methyltransferases and inhibit their function (Egger et al., 2004). Our study strongly suggests that inhibition of DNA methylation and/or DNMT1 protein down-regulation lead(s) to coordinate reactivation of human reelin and GAD67 gene expression. This study also provides evidence that transcription of the human reelin gene is repressed by the methylation-mediated recruitment of DNMT1, MeCP2, and possibly other corepressors, including certain HDACs.

Materials and Methods

Cell Culture and Drug Treatments. NT-2 cells (Stratagene, La Jolla, CA) were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal bovine serum and 1% penicillin, 1% streptomycin, and 1% L-glutamine. DOXO, AZA, and ZEB were obtained from Sigma-Aldrich (St. Louis, MO). Stock solutions of the drugs were prepared by dissolving the substances in either distilled water (DOXO), 50% acetic acid (AZA), or dimethyl sulfoxide (ZEB), and stored at −20°C. For all experiments, control measurements were obtained from vehicle-treated cells. For dose-response quantitative RT-PCR experiments, cells were treated for 48 h with the following concentrations of DOXO; 10, 25, 50, 100, 250, and 1000 nM. Time course mRNA experiments and time course Western blot analyses were carried out after cells had been treated with 100 nM DOXO for 0, 3, 6, 12, 24, 36, or 48 h. For DNA methyltransferase assays and dose-response Western blot analysis, cultures were either untreated or treated with 100 or 250 nM DOXO for 48 h, whereas for cell viability assays, cells were additionally treated with 2 μM DOXO for 48 h. Chromatin immunoprecipitation assays and nonquantitative RT-PCR assay for GAD65 was performed with vehicle-treated cells and cells treated with 250 nM DOXO for 48 h. For both quantitative RT-PCR experiments and Western blot analysis, cells were treated with 5 μM AZA for 48 h and with 500 μM ZEB for either 48 h or 48 h followed by 48-h incubation with untreated medium.

Nuclear Extracts. Nuclear extracts of untreated and treated NT-2 cells were obtained using NE-PER Nuclear and Cytoplasmic Extraction kit as recommended by the manufacturer (Pierce Biotechnology, Rockford, IL). The protein concentrations in the extracts were determined using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA).

Quantitative RT-PCR Analysis. RNA was isolated after ultracentrifugation through CsCl (Chen et al., 2002). Reelin, GAD67, G3PDH, and DNMT1 mRNA contents were measured by competitive RT-PCR with internal standards as described previously (Grayson and Ikonomovic, 1999). Primers were designed to minimally cross at least one exon/intron boundary. For example, the 5′ DNMT1 primer resides in exon 23, whereas the 3′ primer was taken from exon 27 (Ramchandani et al., 1998). For measuring reelin mRNA (GenBank accession number NM_005045), the internal standard was generated by deleting 160 bp in the middle of the 674 amplicon using overlap-extension PCR (Auta et al., 2006). PCR was conducted using the forward primer (+2344) 5′-ATCCGTGGTGCTGAAGTCAGCTTT-3′ and the reverse primer (+3018) 5′-TGAGTACTCCAGCTTCACCTGGTT-3′ (annealing temperature = 68°C, 30 cycles). For GAD67 mRNA (GenBank accession number M81883), the internal standard was generated by deleting 74 bp of the 414-bp amplicon, and the following primers were used for PCR: the forward primer (+1855), 5′-CTTCCAGCCAGACAAGCAGTATGA-3′; and the reverse primer (+2269), 5′-TGGGTTGGAGATGACCATCCGGAA-3′ (annealing temperature = 60°C, 30 cycles). For G3PDH (GenBank accession number BC083511), the internal standard was generated by deleting 216 bp of the 683-bp amplicon, and PCR was carried out using the forward primer (+237) 5′-CTGAGAACGGGAAGCTTGTCATCA-3′ and reverse primer (+920) 5′-TGTCGCTGTTGAAGTCAGAGGAGA-3′ (annealing temperature = 60°C, 30 cycles). For measuring DNMT1 mRNA (GenBank accession number BC092517), the internal standard was generated by deleting 196 bp of the 509-bp amplicon, and PCR was carried out using the forward primer (+2228) 5′-AATCGCATCTCTTGGGTCGGAGAA-3′ and the reverse primer (+2737) 5′-ACGGGCACAGCTCACACAGAATTT-3′ (annealing temperature = 65°C, 30 cycles). The following primers were used for the PCR amplification of GAD65 cDNA (GenBank accession number NM_000818): forward primer, (+989) 5′-TTTCTCTCAAGAAGGGAGCTGCAG-3′; and reverse primer (+1788) 5′-GGGTTGGTAGCTGACCATTGTGG-3′ (annealing temperature = 60°C, 34 cycles).

DNMT Assay. To measure DNA methyl transferase activity, we used a modification of a previously published method (Szyf et al., 1991). A typical methylation reaction (30 μl) contained 1 μg of oligonucleotides [poly(dI-dC)·poly(dI-dC)] (GE Healthcare, Little Chalfont, Buckinghamshire, UK), an appropriate volume of nuclear extract containing 13 μg of protein and 12.2 nM *S*-adenosyl-L-[methyl-^{3}H]methionine (specific activity, 82 Ci/mmol; GE Healthcare) in reaction buffer (20 mM Tris, pH 7.6, 25% glycerol, 10 mM EDTA, 28 mM 2-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride). The reaction mixtures were incubated at 37°C for 3 h, followed by incubation at 65°C for 10 min. Afterward, 1 ml of 10% trichloroacetic acid was added, and samples were incubated overnight at 4°C. Mixtures were then filtered through Whatman GF/C glass microfiber filters and washed twice with 2 ml of trichloroacetic acid. Filters were immersed in 3 ml of scintillation cocktail (Scintiverse; Fisher Scientific, Pittsburgh, PA) for radioactivity counting.

Western Blots. Nuclear extract proteins were separated onto 4 to 20% (DNMT1) or 10 to 20% (MeCP2) Tris-glycine gels and transferred overnight (DNMT1) or for 2 h (MeCP2) to nitrocellulose membranes (Invitrogen, Carlsbad, CA). The membranes were blocked with PBS/Tween 20 (0.1%) containing 5% nonfat dry milk for 1 h followed by an overnight incubation at 4°C with DNMT1 polyclonal antibody (1:1000 dilution; New England Biolabs, Ipswich, MA) or MeCP2 polyclonal antibody (1:500; Abcam, Cambridge, MA). Membranes were then rinsed three times in PBS and incubated with peroxidase-labeled secondary antibody (1:3000; GE Healthcare). Immunoreactive bands were visualized using the enhanced chemiluminescence plus Western blotting detection system (GE Healthcare Bio-Sciences). The intensity of β-actin immunofluorescence was determined on the same blots using β-actin monoclonal antibodies (1:5000 dilution; Sigma-Aldrich), and the corresponding signals were used for a comparative estimation of the amounts of protein applied to the gels. Blots were scanned, and bands were visualized using a Storm 860 PhosphorImager (GE Healthcare). Band intensities were analyzed using ImageQuant software (GE Healthcare).

Chromatin Immunoprecipitation Assays. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit protocol (Upstate Biotechnology, Lake Placid, NY) as described previously (Mitchell et al., 2005). In brief, 10^7 nontreated or DOXO-treated cells were fixed using 1% formaldehyde at room temperature for 10 min. Cells were washed twice in ice-cold PBS, resuspended in SDS-lysis buffer, and sonicated until cross-linked chromatin was sheared to an average DNA fragment length of 200 to 800 bp. The sonicated lysate (5%) was used to quantitate the total amount of DNA present in different samples before immunoprecipitation (inputs). Chromatin preparations were immunoprecipitated using anti-DNMT1 monoclonal antibody (Imgenex, San Diego, CA), anti-MeCP2 and anti-acetyl-histone H3 polyclonal antibodies (Upstate). Nonimmunoprecipitated samples were used as negative controls. Precipitated complexes were bound to protein G-agarose, washed, and then eluted in 1% SDS/0.1 M $NaHCO_3$. Cross-linking between DNA and proteins was reversed by heating the samples at 65°C overnight, followed by Proteinase K digestion at 65°C for 1 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation, and 4 μl of a 20-μl sample was analyzed by PCR. The primers for the reelin promoter region were 5′-CCGGGACACGTGTGGCGGCG-3′ (forward, −220 bp) and 5′-AAAGCGGGGGTAATAGCCAGCCGC-3′ (reverse, +262 bp). The protocol included an initial denaturation cycle (5 min, 94°C), 40 cycles of denaturation (1 min, 94°C), annealing (1 min, 62°C), and extension (1 min, 72°C), followed by the final extension cycle (7 min, 72°C). For the β-globin locus control region, the forward primer (+3961 bp) was 5′-AGACACTTGCTCTTTCCAGGACTT-3′, whereas the reverse primer (+4250 bp) was 5′-TGCCAGTATATGTGCTTCGATAGG-3′. The amplification included an initial denaturation cycle (5 min, 94°C), 40 cycles of denaturation (1 min, 94°C), annealing (1 min, 55°C), and extension (1 min, 72°C), followed by the final extension cycle (7 min, 72°C). PCR amplification products were separated on 1.6% agarose gels, and optical density readings were determined using a computer-assisted densitometry program (Kodak EDAS 290; Eastman Kodak Co., Rochester, NY). For all experiments, input and immunoprecipitated DNA samples were below saturation levels after PCR.

Cell Viability Assays. Cell cultures were treated with vehicle-containing medium or medium supplemented with 100 nM, 250 nM, or 2 μM DOXO. After 48 h, medium was removed and replaced with control medium containing 50 μM propidium iodide (a marker of cell damage) and 1 μM calcein acetoxymethyl ester (a marker of cell viability). After 10 min of incubation, the fluorochrome-containing medium was removed and replaced with control medium, and cell density and viability were examined by fluorescence microscopy.

Statistical Analyses. All experimental results are expressed as mean ± S.E.M. of three independent experiments (a minimum of three separate measurements were obtained per experiment). Student's *t* test (for ChIP results) and one-way ANOVA followed by the Bonferroni multiple comparison test (for all other results) were used to assess significance of the differences between groups. Analyses were conducted using SigmaStat software (Systat Software, Point Richmond, CA). In addition, dose-response curves for reelin and GAD67 gene induction were obtained using Prism version 4 (GraphPad Software, San Diego, CA).

Results

DOXO Increases Reelin and GAD67 Gene Expression in a Similar Dose- and Time-Dependent Manner. The initial step in our study was to explore whether treatment of human neural progenitor (NT-2) cells with DOXO, a drug that acts as a DNMT1 inhibitor, would lead to changes in reelin and GAD67 mRNA expression. In NT-2 cells, background levels of reelin mRNA are barely detectable (0.010 ± 0.0015 pg of reelin mRNA per microgram of total RNA), whereas these cells show significant expression of GAD67 mRNA (0.12 ± 0.013 pg of GAD67 mRNA per microgram of total RNA). We observed a dose-dependent increase in both reelin and GAD67 mRNA levels after 48 h of DOXO treatment (Fig. 1, A and B). The data showed that expression of the reelin mRNA increased up to 92-fold, at which a maximal response was achieved using 250 nM DOXO (0.92 ± 0.15 pg of reelin mRNA per microgram of total RNA). Likewise, 250 nM DOXO led to a 20-fold (maximal) induction of GAD67 mRNA (2.5 ± 0.23 pg of GAD67 mRNA per microgram of total RNA). To demonstrate the specificity of the reelin and GAD67 mRNA induction by DOXO, we examined the expression of two additional genes, G3PDH and GAD65, after the same treatment. In contrast to the changes in reelin and GAD67 mRNAs, G3PDH mRNA levels were not significantly changed independent of the concentration of drug used (Fig. 1, A and B). Likewise, GAD65 mRNA, which is not expressed in NT-2 cells at readily detectable levels, was not induced even with the DOXO treatment that maximally activated reelin and GAD67 mRNAs (Fig. 1D). We next constructed dose-response curves to compare EC_{50} values for reelin and GAD67 mRNA induction in response to DOXO. These analyses revealed the EC_{50} values to be 102 and 103 nM for reelin and GAD67 mRNA increases, respectively (Fig. 1C). Both the maximal induction at 250 nM concentration of the drug together with the nearly equal EC_{50} values for these mRNAs, suggest that reelin and GAD67 are activated in a similar dose-dependent manner.

To explore the time frame in which changes in reelin and GAD67 mRNA expression occur, NT-2 cells were treated with 100 nM DOXO (EC_{50} value) for various lengths of time. Data

from these experiments showed that both reelin and GAD67 mRNA levels were increased in a similar temporal manner as well (Fig. 2, A and B). Most importantly, the initial induction of both mRNAs occurred 12 h after initiating drug treatment (Fig. 2B). As measured by competitive RT-PCR, incubation of NT-2 cells with 100 nM DOXO for 12 h increased reelin mRNA levels 5.3-fold (from 0.019 ± 0.003 to 0.1 ± 0.002 pg of reelin mRNA per microgram of total RNA) and GAD67 mRNA levels 4-fold (from 0.15 ± 0.015 to 0.63 ± 0.057 pg of GAD67 mRNA per microgram of total RNA). Together with the dose-response study, these results suggest that DOXO treatment leads to a coordinated up-regulation of reelin and GAD67 mRNA expression.

Induction of Reelin and GAD67 Genes Is Associated with Reduced DNMT Enzymatic Activity and Decreased DNMT1 Protein Levels. The next goal was to examine whether DOXO inhibits DNA methyltransferase enzyme activity in the same concentration range in which it induces changes in reelin and GAD67 mRNA levels. For this purpose, we used an in vitro enzymatic assay using nuclear extracts prepared from nontreated and DOXO-treated NT-2 cells. Extracts were used to measure methyltransferase activity with [^{3}H]*S*-adenosylmethionine and an artificial DNA substrate. The data showed that 100 nM and 250 nM DOXO treatment of NT-2 cells (48 h) resulted in a significant 70% and 83% inhibition of nuclear DNMT activity, respectively (Fig. 3).

To determine whether the reduction of DNMT enzymatic activity was due, at least in part, to decreased DNMT1 protein levels, we performed Western blot analyses. We observed a significant down-regulation of nuclear DNMT1 protein after 100 and 250 nM DOXO treatments (73% and 83%, respectively, Fig. 4, A and B). Although DNMT1 protein is predominantly localized in the nucleus of NT-2 cells, a small cytoplasmic fraction exists. The cytoplasmic DNMT1 protein showed a similar trend toward decrease after DOXO treatment (data not shown). To assess whether DNMT1 mRNA also showed a similar decrease, we examined the expression of DNMT1 mRNA under the same conditions. The corresponding mRNA was not reduced by 100 and 250 nM DOXO (Fig. 4, C and D), implying that DOXO-induced DNMT1 protein down-regulation is a post-transcriptional event. Because it was of interest to determine whether the decrease in DNMT1 protein levels paralleled temporally the induction of reelin and GAD67 mRNAs, we performed time course Western blot analysis. As shown in Fig. 4, E and F, 100 nM DOXO treatment led to a time-dependent decrease in DNMT1 protein levels. The reduction of DNMT1 protein was apparent as early as 6 h after drug treatment (78% of the control levels),

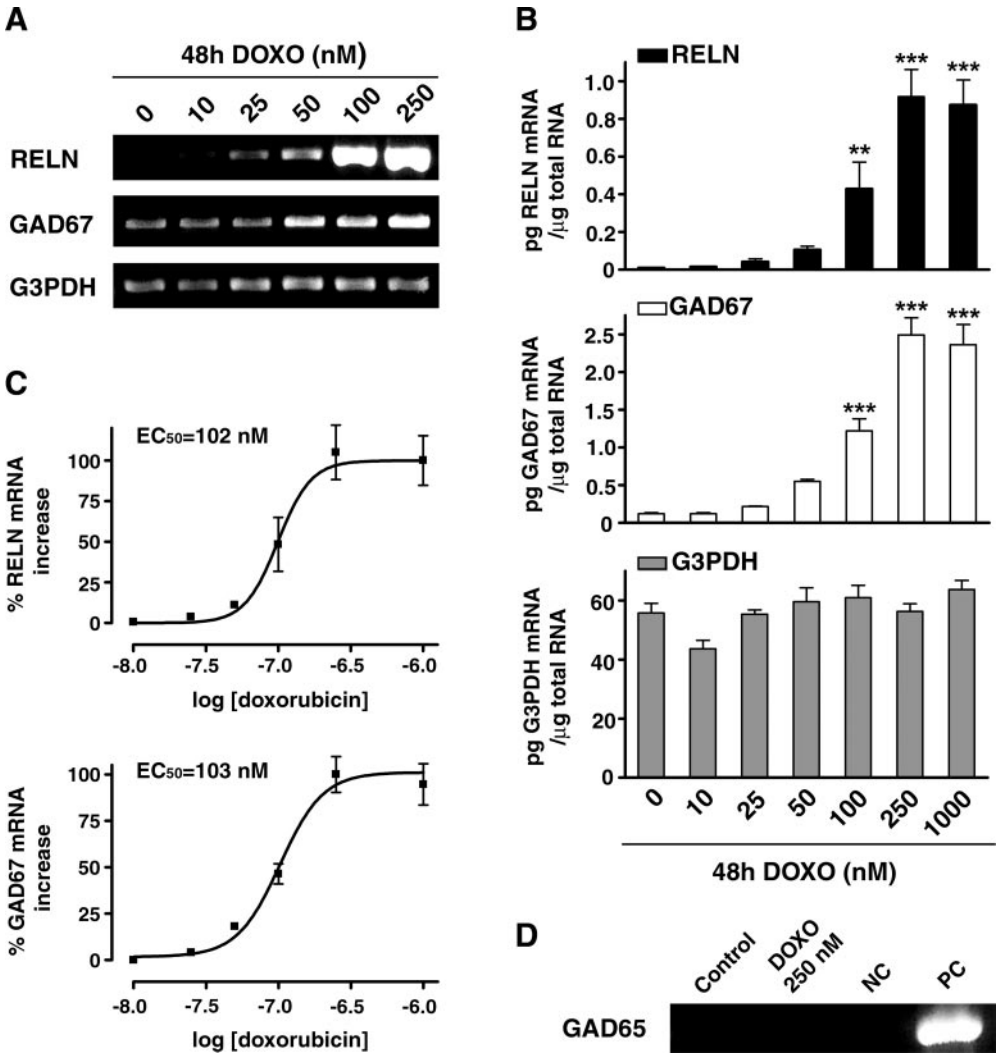

Fig. 1. DOXO treatment leads to dose-dependent increase in reelin and GAD67 mRNA levels. A, representative gels showing typical nonquantitative RT-PCR analysis of reelin, GAD67, and G3PDH mRNAs. B, bars showing results of quantitative analysis of reelin, GAD67, and G3PDH mRNA levels in NT-2 cells treated with different concentrations of DOXO for 48 h. Data are presented as amount (picograms) of reelin, GAD67, or G3PDH mRNA per 1 μg of total RNA at the indicated concentrations of DOXO (*x*-axis). C, dose-response curves for reelin and GAD67 mRNA induction after 48-h DOXO treatment plotted as the log of drug concentration (*x*-axis). To compare reelin and GAD67 dose-response curves, the response is expressed as a percentage of the maximal reelin or GAD67 mRNA increase (*y*-axis). A baseline correction was performed: *y*-axis values are normalized so that the smallest (baseline) mRNA values are defined as 0% response, whereas the highest values (mRNA levels that correspond to maximal gene induction) are defined as 100% response. EC_{50} is the effective concentration of drug that leads to 50% of maximal reelin or GAD67 gene induction. D, representative gel showing RT-PCR analysis of GAD65 mRNA expression in vehicle- or DOXO-treated (250 nM, 48 h) NT-2 cells. RNA isolated from untreated mouse primary neuronal cultures and distilled water were used as positive (PC) and negative controls (NC), respectively. Data represent mean ± S.E.M. ***, $p < 0.001$; **, $p < 0.01$ versus control group (one-way ANOVA followed by Bonferroni test).

whereas the amount of the protein decreased by half after 24 h.

AZA and ZEB Treatments Induce Reelin and GAD67 Gene Expression Associated with DNMT1 Protein Depletion. To confirm that reelin and GAD67 mRNAs are coordinately regulated through methylation-dependent mechanisms, we used two additional methylation inhibitors, AZA and ZEB (Fig. 5, A–D). NT-2 cells were treated with 5 μM AZA for 48 h because it was shown previously that in the same cell system, this treatment leads to a significant increase in reelin mRNA levels (Chen et al., 2002), along with a decrease in reelin promoter methylation (Mitchell et al., 2005). As expected, AZA treatment increased reelin mRNA levels approximately 11-fold (from 0.0095 ± 0.0018 to 0.10 ± 0.015 pg of reelin mRNA per microgram of total RNA). However, in the current study, we showed that the same AZA treatment also led to a 10-fold induction of GAD67 mRNA levels (from 0.12 ± 0.011 to 1.25 ± 0.13 pg of GAD67 mRNA per microgram of total RNA). We also performed a dose-response study with ZEB, treating NT-2 cells with the following concentrations for 48 h: 0.5, 5, 50, and 500 μM. As reported by another group using different cell lines, low concentrations of ZEB failed to induce their gene of interest (Cheng et al., 2004). Likewise, we found no changes in either reelin or GAD67 mRNAs at low concentrations of ZEB (data not shown). However, at 500 μM concentration of ZEB treatment (48 h), the expression of reelin mRNA was increased approximately 9-fold (0.09 ± 0.004 reelin mRNA per microgram of total RNA), whereas GAD67 mRNA levels were elevated 2.5-fold (0.32 ± 0.03 pg of GAD67 mRNA per microgram of total RNA). Because the previous study (Cheng et al., 2004) suggested that ZEB induced the demethylation of the p16 gene with a 2-day delay, we also treated NT-2 cells with 500 μM ZEB for 48 h and then allowed cells to grow in a fresh medium containing no drugs for an additional 48 h before RNA isolation (2 + 2 days). This treatment led to a more significant induction of both genes, with an almost 20-fold increase in reelin (0.19 ± 0.02 pg of reelin mRNA per microgram of total RNA) and 12.5-fold increase in GAD67 mRNA levels (1.6 ± 0.05 pg of GAD67 mRNA per microgram of total RNA). As in the case of DOXO treatment, neither AZA treatment nor ZEB treatment was associated with significant changes in G3PDH mRNA levels (Fig. 5B). However, all three treatments led to an almost complete depletion of DNMT1 protein, as shown by Western blot analysis (Fig. 5, C and D).

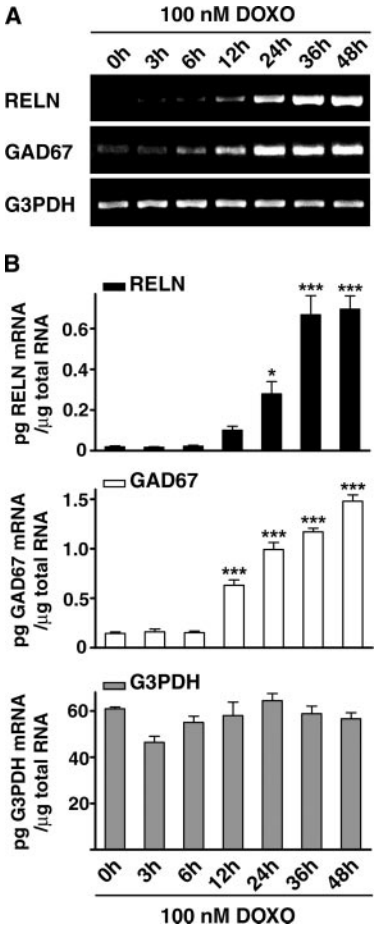

Fig. 2. DOXO treatment induces reelin and GAD67 genes in a time-dependent manner. A, representative gels showing nonquantitative RT-PCR analysis of reelin, GAD67, and G3PDH mRNAs after DOXO treatment for the indicated times. B, bars showing the results of quantitative analysis using competitive RT-PCR and internal standards for the reelin, GAD67, and G3PDH mRNA levels in NT-2 cells treated with 100 nM DOXO for various times. Results are presented as amount (in picograms) of reelin, GAD67, or G3PDH mRNA per 1 μg of total RNA. Data represent mean ± S.E.M. ***, $p < 0.001$; *, $p < 0.05$ versus control group (one-way ANOVA followed by Bonferroni test).

Activation of Reelin mRNA by DOXO Is Accompanied by the Dissociation of DNMT1 and MeCP2 and Increased Histone Acetylation from the Promoter Region. We next sought to understand the mechanisms by which DNMT inhibitors induce the reelin and GAD67 genes. We examined changes at the level of the reelin promoter, because we had established previously *cis*-regulatory elements that are operative in its regulation (Chen et al., 2002). We also have strong evidence showing that the reelin promoter in NT-2 cells is silenced by methylation, whereas the activation of the reelin gene corresponds with a decreased methylation of the promoter. Using ChIP assays, we explored the possibility that inhibition of methylation results in a release of repressor proteins from the reelin promoter. Proteins and DNA were first

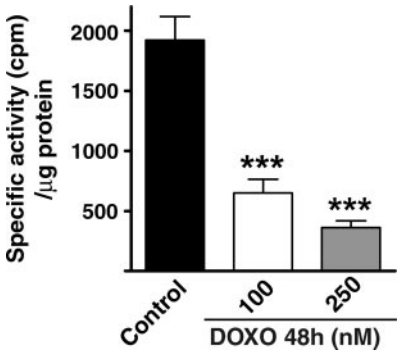

Fig. 3. DOXO reduces the DNA methyltransferase activity of NT-2 cells. Total DNA methyltransferase activity of nuclear extracts from untreated or DOXO-treated calls was assayed in vitro by measuring the incorporation of ^{3}H-labeled methyl group donor *S*-adenosyl-L-methyl-methionine, into the DNA substrate [poly(dI-dC)·poly(dI-dC) oligonucleotide]. Data are expressed as specific radioactivity (total radioactivity − nonspecific radioactivity) normalized to the amount of the protein present in the corresponding nuclear extracts. Data represent mean ± S.E.M. ***, $p < 0.001$ versus control group (one-way ANOVA followed by Bonferroni test).

cross-linked, and chromatin was sonicated to an average DNA fragment size from 200 to 800 bp (Fig. 6A). Using specific antibodies for immunoprecipitation, we examined the association of MeCP2 and DNMT1 proteins with the promoter both before and after 48-h 250 nM DOXO treatment. ChIP data showed that DNMT1 and MeCP2 are bound to the reelin promoter in untreated NT-2 cells (Fig. 6, B and C). In contrast, the induction of reelin mRNA by DOXO corresponded with a dissociation of these proteins from the promoter region (Fig. 6, B and C). By using acetyl H3 pull-down assays, we further explored whether changes in DNMT1 and MeCP2 binding were accompanied by changes in the acetylation status of histone H3 in the vicinity of the promoter. As shown (Fig. 6, B and C), DOXO significantly increased the amount of acetyl H3 histone associated with the same region, implying that this treatment also alters chromatin structure in the vicinity of the reelin promoter. The specificity of these changes with the reelin promoter was demonstrated by amplifying the β-globin control region in parallel after pull-down assays using the same antibodies. As expected for a gene that is not epigenetically regulated, none of the examined proteins was bound to the β-globin control region either before or after DOXO treatment (Fig. 6B). Lack of DNMT1 association with the reelin promoter correlated with almost complete depletion of DNMT1 protein from NT-2 cell nuclear extracts after 48-h 250 nM DOXO treatment. However, neither 100 nor 250 nM 48-h DOXO treatment led to significant changes in MeCP2 protein levels (Fig. 6, D and E).

DOXO Treatments (100 and 250 nM for 48 Hours) Are Not Associated with Significant NT-2 Cell Loss. Besides acting as a DNMT1 inhibitor, DOXO can act as a DNA-damaging agent that activates p53 and induces apoptosis (Esteve et al., 2005). Previous studies using HCT116 cells showed that only at a 1×10^{-6} M concentration (and not lower), DOXO induced significant cell death that was related to apoptosis (Yokochi and Robertson, 2004). Here we report that maximal induction of reelin and GAD67 genes was associated with 250 nM DOXO. Compared with EC_{100} treatment, the 1 μM treatment was associated with slightly reduced reelin and GAD67 mRNA levels (Fig. 1B), which could be explained by the effect of DNA damage at that concentration of DOXO. To confirm that reelin and GAD67 gene induction is not related to apoptosis, we performed cell viability assays after 100 nM, 250 nM, and 2 μM (48 h) DOXO treatments. As shown in Fig. 7, 100 and 250 nM DOXO treatments were not associated with significant NT-2 cell death. However as anticipated, 2 μM DOXO induced considerable cell loss due to apoptosis and possibly to necrosis.

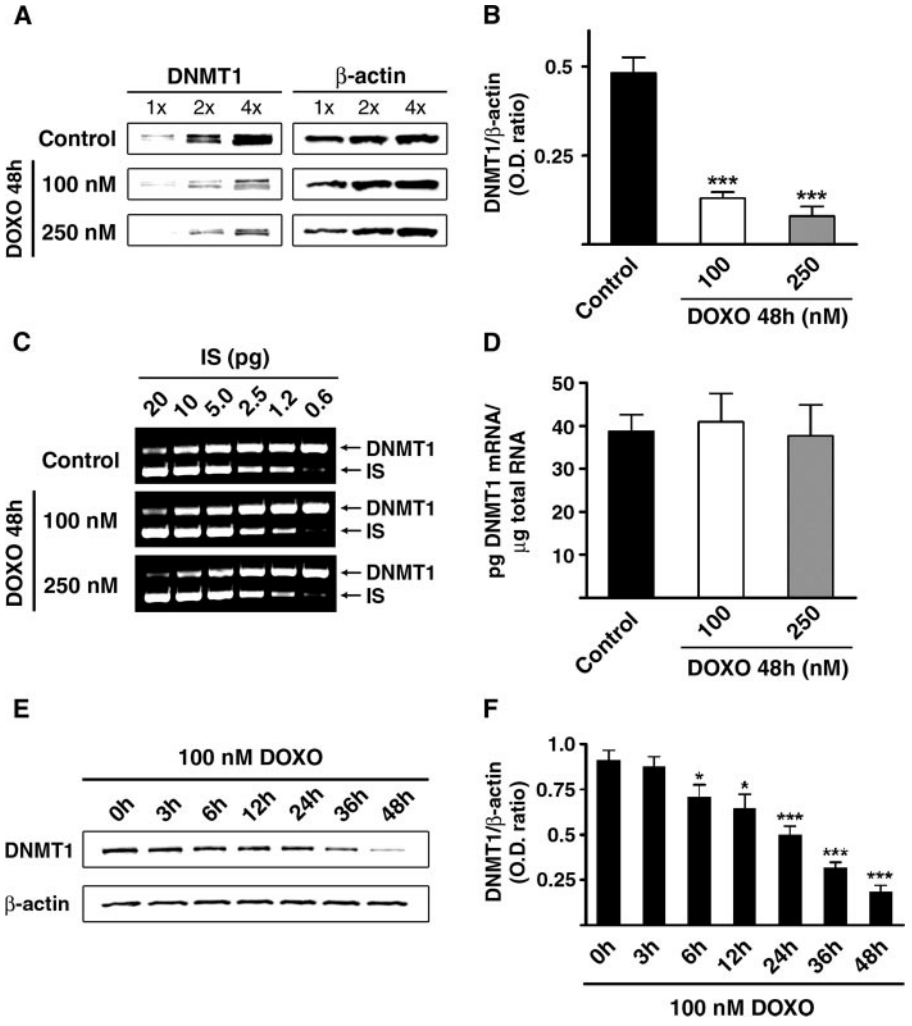

Fig. 4. DOXO down-regulates DNMT1 protein levels post-transcriptionally. The representative Western immunoblots and the ratio of the DNMT1 band over the area of the β-actin band in nuclear extract protein samples from untreated cells (control) and cells treated with either 100 and 250 nM DOXO for 48h (A and B) or 100 nM DOXO for various times (E and F); 1× corresponds to 2.5 μg of protein; time course analysis was done using 5 μg of protein. C, representative RT-PCR gels with internal standards, and D, bars showing DNMT1 mRNA levels in vehicle-treated and DOXO-treated cells, obtained using competitive RT-PCR assay. In each tube, competition was carried out among various concentrations of the DNMT1 internal standard (IS) and 100 ng of total RNA extracted from untreated cells (control) or cells treated with 100 and 250 nM DOXO for 48 h. Data are presented as amount of DNMT1 mRNA (in picograms) per 1 μg of total RNA. Data represent mean ± S.E.M. ***, $p < 0.001$; *, $p < 0.05$ versus control group (one-way ANOVA followed by Bonferroni test).

Discussion

The data presented here clearly demonstrate that both reelin and GAD67 mRNA expression were significantly induced by three different DNMT inhibitors, namely DOXO, AZA, and ZEB. Most importantly, the detailed study with DOXO showed that this induction occurs 1) in a similar dose-dependent manner (as shown by the same EC_{50} and EC_{100} values for the induction of both mRNAs) and 2) within the same time frame (both mRNAs begin to be induced after ~12 h). The similar concentration-dependent and temporal activation patterns of the reelin and GAD67 mRNAs strongly support our hypothesis that these two genes are coordinately regulated. Moreover, the finding that DOXO inhibits DNA methyltransferase activity in the same concentration range that induces reelin and GAD67 mRNA expression provides additional evidence that reelin and GAD67 genes are activated epigenetically. However, the assay we used measures total DNA methyltransferase activity and probably reflects the activities of so-called maintenance methyltransferase (DNMT1) and the activity of de novo methyltransferases (DNMT3A and DNMT3B). Using recombinant DNMT1 protein, Yokochi and Robertson (2004) previously showed that DOXO inhibits the enzymatic activity of DNMT1. As indirect evidence that this drug inhibits DNMT1 enzymatic activity under the conditions that we applied, we showed that the reduction of total DNMT activity is highly correlated with the reduction in DNMT1 protein levels following the same DOXO treatment. Furthermore, AZA and ZEB treatments, which induced reelin and GAD67 mRNAs, led to a complete depletion of nuclear DNMT1 protein. Consistent with this, a previous study of ours demonstrated that the knockdown of DNMT1 protein is associated with an up-regulation of mouse reelin and GAD67 mRNA levels in cortical neurons in vitro (Noh et al., 2005). Taken together, these data imply a possible role for DNMT1 in the coordinated regulation of the reelin and GAD67 genes. Having said this, we cannot exclude the possibility that DNMT3A and/or 3B might also play a role in these events.

In dividing cells such as NT-2 cells, DNMT1 is believed to be mainly involved in the methylation of hemimethylated DNA. This process predominates during DNA replication. Therefore, drugs that inhibit DNMT1 enzymatic activity, such as DOXO, AZA, and ZEB, may require several cell divisions to induce significant changes in promoter methylation status, along with changes in mRNA expression (Egger et al., 2004). It is interesting that we observed an induction of

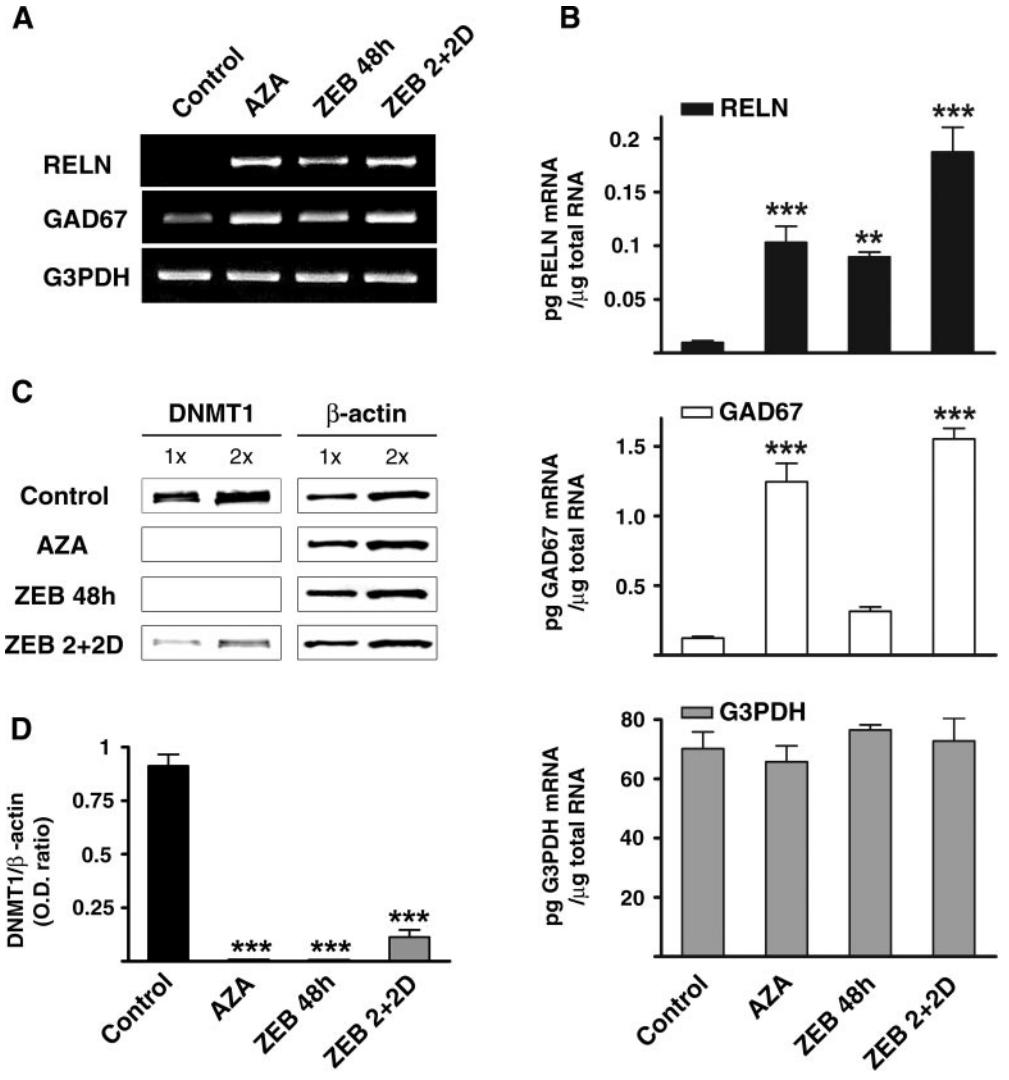

Fig. 5. The effects of AZA and ZEB treatments on reelin and GAD67 mRNA expression and DNMT1 protein levels. A, representative gels of nonquantitative RT-PCR analysis and B, bars showing the results of quantitative analysis of the reelin, GAD67, and G3PDH mRNA levels in NT-2 cells treated with 5 μM AZA for 48 h and 500 μM ZEB for either 48 h (ZEB 48 h) or 48 h followed by 48-h incubation with untreated medium (ZEB 2 + 2D). Results are presented as the amount (in picograms) of reelin, GAD67, or G3PDH mRNA per microgram of total RNA. C, representative DNMT1 and β-actin Western immunoblots, and D, the ratio of the DNMT1 band over the area of the β-actin band in nuclear extract protein samples of control and AZA- or ZEB-treated NT-2 cells (1× corresponds to 5 μg of protein). Data represent mean ± S.E.M. ***, $p < 0.001$; **, $p < 0.01$ versus control group (one-way ANOVA followed by Bonferroni test).

both reelin and GAD67 mRNAs as early as 12 h after initiating DOXO treatment. In contrast, 20 to 24 h are needed to complete one cell cycle. This observation led us to consider a possible corepressor role of DNMT1 protein in regulating reelin and GAD67 mRNA expression. It has been shown that DNMT1 represses gene transcription through its noncatalytic domain independent of its methyltransferase function. This action occurs through the recruitment of MeCP2 and HDACs (Fuks et al., 2000; Burgers et al., 2002; Kimura and Shiota, 2003). As an example, it has been shown recently that DNMT1 can suppress the activity of the metallothionein-I gene promoter regardless of its methylation status (Majumder et al., 2006). To explore whether this is also the case with the reelin and GAD67 genes, we first checked the time frame of DNMT1 protein down-regulation. It is striking that DNMT1 protein levels start to decrease 6 h before the induction of reelin and GAD67 mRNAs occurs. Our data demonstrated that the down-regulation of DNMT1 occurs post-transcriptionally, because we show that DOXO does not induce changes in DNMT1 mRNA levels. It seems likely that DNMT1 may get trapped in a DOXO-DNA complex, which subsequently targets DNMT1 for degradation (Yokochi and Robertson, 2004). This event can be replication-independent, because it has been shown that in addition to S phase, DNMT1 is continuously loaded onto chromatin during the G_2 and M phases of the cell cycle (Easwaran et al., 2004). In support of our finding, another group reported that 2 h of AZA treatment is sufficient to induce significant replication-independent reduction in DNMT1 protein levels (Ghoshal et al., 2005). Furthermore, we demonstrated that DNMT1 protein decreases in a time-dependent fashion, very similar to that seen for the increases in reelin and GAD67 mRNA levels.

As additional evidence for the corepressor role of DNMT1, we showed (using ChIP assays) that this protein is bound to the reelin promoter when the gene is silent or transcriptionally inactive. In contrast, the maximal induction of the reelin mRNA is accompanied by a complete dissociation of DNMT1 from the promoter regulatory region. These data suggest that DNMT1 is involved in keeping the reelin promoter in a repressed state in NT-2 cells. It seems likely that the slight activation of the reelin (and probably GAD67) gene(s), seen 12 h after beginning DOXO treatment, is triggered by decreased amounts of DNMT1 in these cells and the subsequent decreased binding of DNMT1 to the reelin promoter. This, in turn, might lead to the release of the repressor complex from the reelin promoter. Evidence suggests that this repressor complex probably includes MeCP2 and certain HDACs. It has been shown recently that the down-regulation of reelin and GAD67 mRNAs corresponds with increased recruitment of MeCP2 to the mouse reelin and GAD67 promoters (Dong et al., 2005). Here we demonstrate that the maximal activation

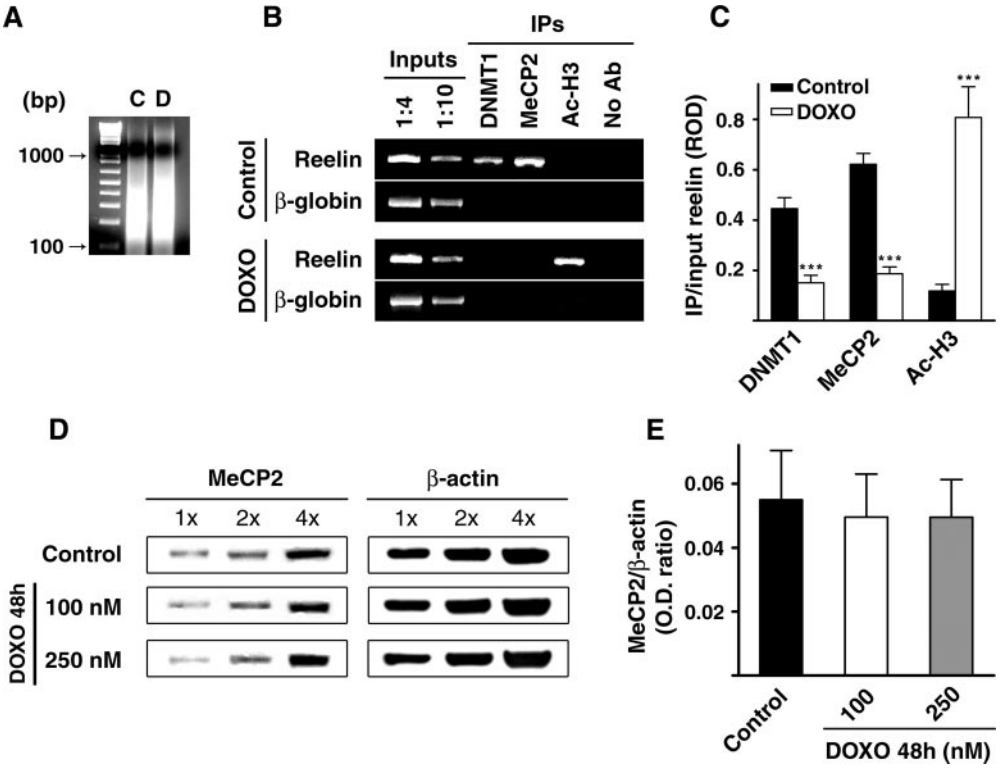

Fig. 6. DOXO induces dissociation of DNMT1 and MeCP2 and increased acetylation of H3 histones in the reelin promoter region. A, chromatin samples containing 200- to 800-bp DNA fragments were generated from cells treated with either control medium (C, control) or 250 nM DOXO for 48 h (D, DOXO). B, for both treatments, the reelin promoter region (482-bp band) and β-globin gene fragments (289-bp band) were PCR-amplified from nonimmunoprecipitated input (1:10 and 1:4 dilutions), samples immunoprecipitated with DNMT1 antibody (DNMT1 IP), MeCP2 antibody (MeCP2 IP) or anti-acetyl histone H3 antibody (Ac-H3 IP), and negative control (no antibody). C, results of semiquantitative analysis of the occupancy of DNMT1 and MeCP2 to the reelin promoter in vehicle- and DOXO-treated cells normalized to input DNA (1:4 dilution). For comparison, the amounts of acetylated histone H3 is shown after treatment. Data are presented as a ratio of relative optical densities (ROD) of the bands within the immunoprecipitated sample (IP) and input lanes derived from ethidium bromide-stained gels. Data represent mean ± S.E.M. ***, $p < 0.001$. Control versus DOXO (Student's t test). D, representative MeCP2 and β-actin Western immunoblots, and E, the ratio of the MeCP2 band over the area of the β-actin band from nuclear extract protein samples of vehicle-treated (control) and DOXO-treated NT-2 cells (1× corresponds to 5 μg of protein). Data represent mean ± S.E.M. (one-way ANOVA followed by Bonferroni test).

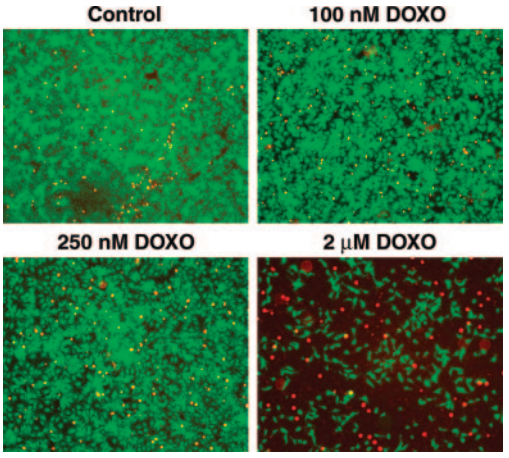

Fig. 7. DOXO toxicity in NT-2 cell culture. Cell cultures were either vehicle-treated (control) or treated with 100 nM (EC_{50} value), 250 nM (EC_{100} value), and 2 μM DOXO for 48 h. Cell density and viability were examined by fluorescence microscopy after the incubation with medium containing calcein-acetoxymethyl ester (green, live cells) and propidium iodide (red, dead cells) (magnification, 5×).

of the reelin gene by DOXO is associated with the dissociation of MeCP2 from the promoter region and an increase in H3 histone acetylation in the vicinity of the promoter. Because MeCP2 binds specifically to methylated cytosines, our data also suggest that 48 h of DOXO treatment induces changes in the methylation status of the reelin promoter. This conclusion is strengthened by the finding that unlike DNMT1, MeCP2 protein levels did not change after the same treatment. We have shown previously that all treatments that induce reelin expression, including AZA, also decrease reelin promoter methylation (Chen et al., 2002; Mitchell et al., 2005). However, as noted previously, 12-h treatment that produces a slight induction of reelin and GAD67 mRNAs seems likely to be insufficient to induce changes in promoter methylation status. Therefore, we suggest that promoter methylation per se may not be sufficient to keep the reelin promoter in a fully repressed state. Complete silencing of the reelin promoter probably requires the fully assembled repressor complex and highly condensed chromatin maintained by the recruitment of DNMT1. However, we believe that the reelin promoter must be demethylated for the maximal activation of the gene to occur. Additional studies are needed to confirm this speculation.

In conclusion, we highlight several implications of these data. First, the study suggests a mechanism by which reelin and GAD67 mRNAs might be coordinately regulated in GABAergic neurons of the adult brain. It seems likely that both genes may be regulated by methylation of the corresponding promoters. DNMT1 probably has a dual role in this process. One could be its well-established enzymatic (DNA methyltransferase) role, by which it controls the methylation status and the activity level of the reelin, GAD67, and possibly other epigenetically regulated promoters. Another role of DNMT1 could be to participate in the formation of the transcriptional repressor complex by recruiting MeCP2, HDACs, and other corepressors. This may lead to the generation of a more condensed chromatin structure that subsequently limits promoter accessibility. As mentioned above, although we have focused on the role of DNMT1 in this process, we cannot exclude a contributing role for either DNMT 3A and or DNMT 3B.

Second, this study gives new insight into the molecular mechanisms that underlie the down-regulation of reelin and GAD67 mRNAs in the brains of patients with schizophrenia. We propose that the reported up-regulation of DNMT1 (Veldic et al., 2004) leads to the hypermethylation and increased binding of DNMT1 to the reelin and GAD67 promoters. Furthermore, we suggest that there is a subsequent increased recruitment of MeCP2, HDACs, and possibly additional corepressor proteins. However, studies with postmortem human brains will be necessary to confirm this hypothesis. Third, we suggest a new approach in the treatment of schizophrenia that focuses on the reactivation of expression of genes that are down-regulated due to modifications in the epigenome. Thus far, epigenetic drugs (DNA methylation inhibitors and HDAC inhibitors) have been used in cancer treatment, because they often selectively reactivate tumor suppressor genes that are silenced by CpG island promoter methylation (Egger et al., 2004). It seems likely that this may be one of the mechanisms that contributes to the therapeutic benefits of DOXO in some types of cancer. However, of specific interest in the context of schizophrenia research, we report that DOXO concentrations that do not induce significant cell death lead to a robust induction of the reelin and GAD67 mRNAs. Furthermore, our data suggest that DOXO induces changes in the methylation status of the reelin promoter, which has been shown to be hypermethylated in the brains of patients with schizophrenia (Abdolmaleky et al., 2005; Grayson et al., 2005). Although this remains to be addressed experimentally, the changes in methylation most likely occur only at specific promoters, because another group reported no changes in global methylation of genomic DNA after DOXO treatment (Yokochi and Robertson, 2004). We propose that drugs that induce promoter hypomethylation and/or DNMT1 down-regulation might be useful in correcting the reelin and GAD67 mRNA insufficiencies associated with schizophrenia. This means that DNMT1 and HDACs may represent possible new molecular targets to treat patients with schizophrenia. At the same time, because many of these drugs are toxic to cells and may have global effects in the nervous system that have yet to be determined, the safety of these compounds needs to be fully tested in animal models before adopting their use in humans.

Acknowledgments

We dedicate this article to the memory of our close friend and scientific colleague Robert H. Costa, who died September 1, 2006, from complications associated with pancreatic cancer.

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