

Specificity Protein 1 (Sp1)-dependent Activation of the Synapsin I Gene (*SYN1*) Is Modulated by RE1-silencing Transcription Factor (REST) and 5'-Cytosine-Phosphoguanine (CpG) Methylation*

Received for publication, July 10, 2012, and in revised form, December 17, 2012. Published, JBC Papers in Press, December 18, 2012, DOI 10.1074/jbc.M112.399782

Francesco Paonessa, Shahrzad Latifi, Helena Scarongella, Fabrizia Cesca¹, and Fabio Benfenati^{1,2}

From the Department of Neuroscience and Brain Technologies, Istituto Italiano di Tecnologia, via Morego 30, 16163 Genova, Italy

Background: Syn I plays a key role at presynaptic terminals.

Results: Sp1 binds to the *SYN1* promoter, activating its transcription.

Conclusion: Sp1 is a novel regulator of *SYN1* transcription, whose activity is inhibited by REST and CpG methylation.

Significance: Elucidating the mechanisms underlying basal activation of neuron-specific genes is fundamental to understand brain pathologies, where transcription is often dysregulated.

The development and function of the nervous system are directly dependent on a well defined pattern of gene expression. Indeed, perturbation of transcriptional activity or epigenetic modifications of chromatin can dramatically influence neuronal phenotypes. The phosphoprotein synapsin I (Syn I) plays a crucial role during axonogenesis and synaptogenesis as well as in synaptic transmission and plasticity of mature neurons. Abnormalities in *SYN1* gene expression have been linked to important neuropsychiatric disorders, such as epilepsy and autism. *SYN1* gene transcription is suppressed in non-neural tissues by the RE1-silencing transcription factor (REST); however, the molecular mechanisms that allow the constitutive expression of this genetic region in neurons have not been clarified yet. Herein we demonstrate that a conserved region of human and mouse *SYN1* promoters contains *cis*-sites for the transcriptional activator Sp1 in close proximity to REST binding motifs. Through a series of functional assays, we demonstrate a physical interaction of Sp1 on the *SYN1* promoter and show that REST directly inhibits Sp1-mediated transcription, resulting in *SYN1* down-regulation. Upon differentiation of neuroblastoma Neuro2a cells, we observe a decrease in endogenous REST and a higher stability of Sp1 on target GC boxes, resulting in an increase of *SYN1* transcription. Moreover, methylation of Sp1 *cis*-sites in the *SYN1* promoter region could provide an additional level of transcriptional regulation. Our results introduce Sp1 as a fundamental activator of basal *SYN1* gene expression, whose activity is modulated by the neural master regulator REST and CpG methylation.

Brain development and function rely on a highly specific, spatially and temporally controlled pattern of gene expression (1–4). Such a fine regulation of transcription bestows neuronal circuits the ability to fulfill higher functions such as learning and memory. Proper gene expression is maintained through the balancing effects of activating and repressing transcriptional factors. In addition, epigenetic modifications, such as DNA methylation, are emerging as crucial determinants of cell type-specific patterns of transcription (5, 6). Dysregulation of the transcriptional machinery has been linked to several brain pathologies, such as Huntington disease, temporal lobe epilepsy, and dementia (7, 8). Understanding the events triggering neuron-specific gene expression is therefore instrumental to achieve a better understanding of the molecular basis of several neuropathologies.

Synapsin I (Syn I)³ is a key neuronal phosphoprotein that drives the formation, maintenance, and rearrangement of synaptic contacts in neural circuits (9). *SYN1* gene expression gradually increases during brain development and reaches its highest levels in adult neural tissue (10). Syn I knock-out mice develop defects in neuronal plasticity associated with epileptic seizures and behavioral impairments (11–14). Moreover, a nonsense mutation in the *SYN1* open reading frame (ORF) associated with epilepsy and autism has been recently described that causes defects in neurite outgrowth and mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/Erk) phosphorylation, leading to impaired synaptic function (15). The *SYN1* gene is phylogenetically conserved and has been mapped on chromosome X in human, mouse, and other mammals (16). The human and mouse *SYN1* promoter region is a GC-rich sequence without TATA or CAAT elements (17) whose neuron-specific expression is dependent on a highly

* This study was supported by research grants from the Italian Ministry of University and Research (PRIN) and the Compagnia di San Paolo, Torino, Italy. The support of Telethon-Italy (Grant GGP09134) is also acknowledged.

⌘ Author's Choice—Final version full access.

¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed: Istituto Italiano di Tecnologia Via Morego, 30 – 16163 Genova, Italy. Tel.: 39-01071781434; Fax: 39-01071781230; E-mail: fabio.benfenati@iit.it.

³ The abbreviations used are: Syn I, mouse and human synapsin I protein; DIV, days *in vitro*; REST/NRSF, RE1-silencing transcription factor/neuron-restrictive silencer factor; Sp1, specificity protein 1; N2a, Neuro2a; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol; qRT-PCR, quantitative RT-PCR; ANOVA, analysis of variance; CpG, 5'-cytosine-phosphoguanine; 5-AzaC, 5-azacytidine; MBD, methyl CpG binding domain; m, mouse; h, human.

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conserved sequence proximal to the transcriptional start site, containing consensus sites for the RE1-silencing transcription factor/neuron-restrictive silencer factor (REST/NRSF, henceforth referred to as REST). REST is a well known repressor factor that binds a specific 21-bp *cis*-site (RE1) (18) and mediates transcriptional silencing by the recruitment of two distinct co-repressors, mSin3a and CoREST1 (19, 20), which induce histone deacetylation and methylation (21). REST expression significantly decreases during neuronal development; however, it is still present in the adult brain where it represses, but does not totally silence, the expression of specific genes such as ion channels, neurotransmitter receptors, adhesion molecules, and neurotrophins (22, 23). Several studies have shown that REST, by maintaining a constant wrapping of DNA around deacetylated histones, can keep the *SYN1* promoter region in a repressed state, thereby inhibiting the binding of positive factors in undifferentiated neurons and non-neural cells (17). Much information is available about the silencing mechanisms of *SYN1* promoter; however, little is known about the molecular events that drive *SYN1* transcription in adult neurons.

To further characterize the mechanisms that drive constitutive *SYN1* transcription in the adult brain, we subjected the human and mouse gene promoters to a bioinformatic analysis, which predicted a number of *cis*-sites for the ubiquitous protein Sp1 in close proximity to REST binding sites. Sp1 is a ubiquitous zinc-finger protein that binds GC-rich elements (24) in the promoters of many housekeeping as well as tissue-specific genes. After the interaction with target GC boxes, Sp1 stimulates transcription initiation of TATA-less promoters through the recruitment of the TATA box binding protein-transcription factor II D (TBP-TFIID) complex (25). Herein, we demonstrate that Sp1 can directly interact with the human and mouse *SYN1* promoter and that it plays an important role in promoting *SYN1* transcription. Moreover, we report a strict functional interplay between Sp1 and REST, which exerts a dominant negative role on Sp1 function and chromatin affinity on the *SYN1* promoter. Finally, we propose DNA methylation as an additional level of *SYN1* transcriptional regulation. Altogether, our results introduce Sp1 as a fundamental regulator of basal Syn I expression in neural cells.

EXPERIMENTAL PROCEDURES

Materials—All biochemical reagents and drugs were from Sigma-Aldrich, unless otherwise specified. Tissue culture reagents and media were from Gibco-Invitrogen (Life Technologies Corp.) or Sigma-Aldrich.

Antibodies—Fluorescently conjugated secondary antibodies for Western blot analysis were ECL PlexTM goat α -rabbit IgG-Cy5 PA45012 and ECL PlexTM goat α -mouse IgG-Cy3 PA43010 (GE Healthcare). Fluorescently conjugated secondary antibodies for immunofluorescence were from Molecular Probes (Invitrogen). The following primary antibodies were used: polyclonal anti-Sp1 PEP2 (sc-59; Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal anti-REST (07-579; Millipore, Billerica, MA), polyclonal anti-Syn I (ab8; Millipore), ChIPAb+ REST (17-641; Millipore), anti-GAPDH (SAB3500247; Sigma-Aldrich), and polyclonal anti-neuronal class III β -tubulin (T2200; Sigma-Aldrich).

Cell Lines and Cortical Neuron Cultures—Human SH-SY5Y and murine Neuro2a (N2a) neuroblastoma cells, and RAW 264.7 murine macrophages, were cultured in DMEM supplemented with 10% fetal bovine serum, glutamine (2 mM), and antibiotics, in a humidified 5% CO₂ atmosphere at 37 °C. For differentiation, SH-SY5Y and N2a cells were seeded at 60% confluence, and culture medium was replaced after 24 h with DMEM + 1% FBS + 20 μ M retinoic acid (RA). Differentiating medium was replaced every 24 h, for 3 days. In demethylation assays, cells were treated for 48 h with 3 μ M 5-azacytidine, replacing the medium every 24 h. Cortical neurons were dissected from embryonic day 18 mouse C57/BL6 embryos and cultured as described previously (15). Neural precursors were prepared as described previously. Briefly, cells were isolated from the dentate gyrus of adult (6–8 weeks) mice and cultured as monolayer on poly-D-lysine- and laminin-coated flasks in Neurobasal medium containing B27, GlutaMAX, and penicillin-streptomycin solution supplemented with recombinant fibroblast growth factor-2 and epidermal growth factor (26, 27).

Protein Extraction and Western Blotting—Cytoplasmic and nuclear extracts were prepared as follows. The pellet from 5×10^6 cells was resuspended in 400 μ l of Cytoplasmic Buffer (10 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, protease inhibitor mixture (Complete protease inhibitor mixture tablets; Roche Applied Science, Basel, Switzerland) and incubated 20 min on ice with constant shaking. After the addition of 25 μ l of 10% IGEPAL, the solution was briefly vortexed and centrifuged 2 min at $8000 \times g$ at 4 °C. The supernatant containing the cytoplasmic extracts was collected, and the pelleted nuclei were suspended in 50 μ l of Nuclear Buffer (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 0.2 mM EDTA, 25% glycerol, protease inhibitor mixture) and incubated 20 min on ice with constant shaking. Nuclei were centrifuged 25 min at $10,000 \times g$ at 4 °C, and the supernatant (nuclear extract) was collected. The final protein concentration was quantified by using the Bradford protein assay (Bio-Rad). SDS-PAGE and Western blotting were performed by using precast 10% NuPAGE[®] Novex[®] Bis-Tris Gels (Invitrogen). After incubation with primary antibodies, membranes were incubated with fluorescently conjugated secondary antibodies and revealed by a Typhoon TRIO+ variable mode imager (GE Healthcare).

Oligonucleotides and Electrophoretic Mobility Shift Assay (EMSA)—Human (–371 to –295) and mouse (–428 to –319) *SYN1* gene promoter sequences were chemically synthesized (Sigma). Fragments were end-labeled with biotin using the biotin 3' end labeling kit (Pierce). For each gel-shift reaction, a total of 20 nM biotin-labeled probe was dissolved in Binding Buffer (100 mM HEPES, pH 7.2, 400 mM KCl, 30 mM MgCl₂, 10 mM DTT, 50% glycerol) with 500 ng of nuclear extracts and 100 ng of poly(dI-dC). For supershift assays, 1 μ g of anti-Sp1 PEP2 or anti-REST antibodies was preincubated 20 min at room temperature with nuclear extracts before the addition of the labeled probes. The reaction mixture was resolved on a nondenaturing 6% polyacrylamide gel, and the signal of the biotin-labeled DNA was detected by using the LightShift chemiluminescent EMSA kit (Pierce).

Chromatin Immunoprecipitation (ChIP)— 10×10^6 cells were incubated 10 min in 10 ml of DMEM + 1% formaldehyde

before 1 ml of 1.25 M glycine was added. After 5 min, cells were washed twice with cold PBS and collected by centrifugation. The pellet was suspended in 300 μ l of Swelling Buffer (25 mM Hepes, pH 7.8, 1.5 mM MgCl₂, 10 mM KCl, 0.1% IGEPAL, 1 mM DTT, 0.5% PMSF, protease inhibitor mixture), incubated 10 min on ice, briefly vortexed, and centrifuged 5 min at 2000 \times g, at 4 °C. The supernatant was removed, and the nuclear pellet was suspended in 200 μ l of Nuclei Lysis Buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, protease inhibitor mixture) and incubated 10 min on ice. DNA was fragmented by sonication and centrifuged 15 min at 10,000 \times g, at 4 °C. Formaldehyde-fixed DNA-protein complex (100 μ l) was added to 900 μ l of Immunoprecipitation Dilution Buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 1 mM PMSF, protease inhibitor mixture) and incubated with 2 μ g of specific antibody (anti-Sp1 or ChIPAb+ REST) or anti-rabbit IgG antibodies overnight at 4 °C. The day after, 20 μ l of protein A/G agarose (GE Healthcare) were added, and the samples were incubated 1 h at 4 °C. An aliquot from the control condition (100 μ l; no antibody) was saved and used as input. Samples were washed twice with 1 ml of Wash Buffer A (50 mM Hepes, pH 7.9, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF), twice with 1 ml of Wash Buffer B (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.5% IGEPAL, 0.5% sodium deoxycholate, 0.5 mM PMSF), and twice with 1 ml of TE Buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Beads were incubated overnight at 65 °C in 100 μ l of Elution Buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS, 50 mM NaHCO₃, 0.2 M NaCl). De-cross-linked DNA was treated with 40 μ g/ml RNase A (Qiagen, Dusseldorf, Germany) and 20 μ g/ml proteinase K (Sigma) and purified using the PCR purification Kit (Qiagen). The efficiency of chromatin immunoprecipitation was quantified bases on a standard curve prepared using 1% input chromatin. The regions of interest were amplified using the following primers: human SYN1-ChIP 5'-ACC CAA GTG TCC ACC TCC GCT-3' (forward) and 5'-GTG ACG TCA GCG CGC CTT CA-3' (reverse); mouse Syn1-ChIP 5'-GGA GTT TCG TTA CTA CAG GTC CGG A-3' (forward) and 5'-AGC ATG GAC GCG ACC AAG GC-3' (reverse).

Plasmids and Transfections—pN3-Sp1 FL vector was a kind gift of G. Suske (Philipps University Marburg, Marburg, Germany). pN3-Sp3FL was obtained from Addgene (plasmid 24541). Expression vectors encoding for REST and shREST were a kind gift of Dr. J. Meldolesi (Milan, Italy). siRNA against human and murine Sp1 were from Santa Cruz Biotechnology (Sp1 siRNA (m), sc-29488; Sp1 siRNA (h), sc-29487), scramble siRNA-A was used in the control conditions (siRNA-A, sc-37007; Santa Cruz Biotechnology). Human (pGL3-SYN1h) and mouse (pGL3-Syn1m) reporter plasmids were obtained by cloning the sequence of the human (-914 to +11) or murine (-720 to +15) Syn I promoters into the pGL3 luciferase reporter vector (Promega, Madison, WI) within the XhoI and HindIII sites using the following primers: HumanSYN1-FW (XhoI), 5'-TAC TCG AG C CTA GAT TGG CGT GTG TTC TG-3'; HumanSYN1-RV (HindIII), 5'-GGA AGC TT A GGT AGT TCA TGG CTG CGA C-3'; MouseSyn1-FW (XhoI), 5'-CCC TCG AGC TTT TCT TTG CCC GAC AGA G-3';

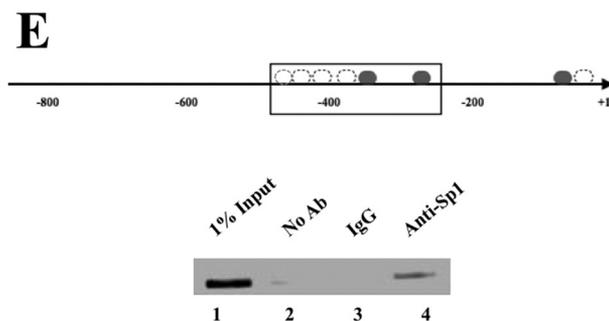
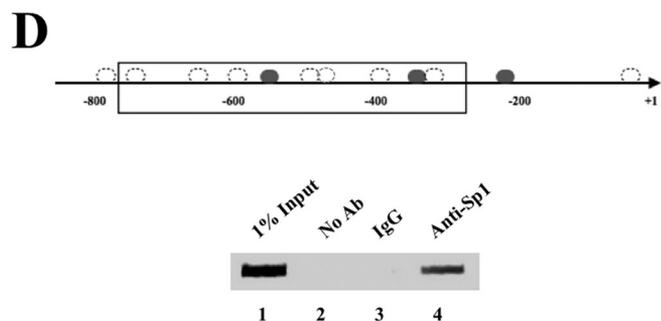
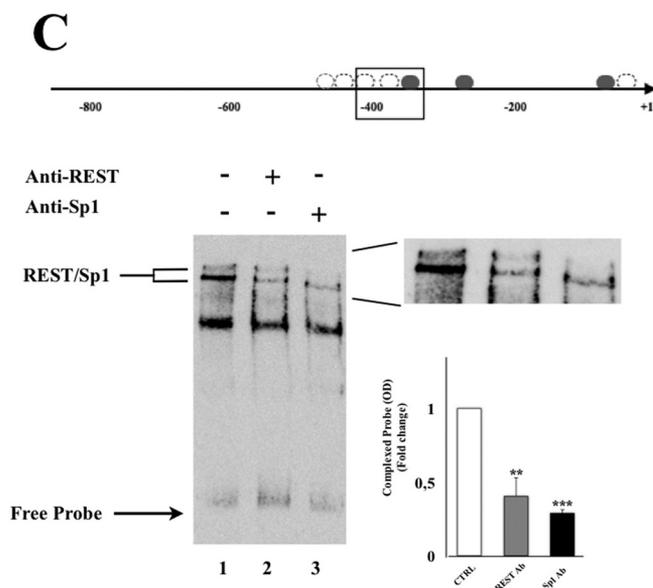
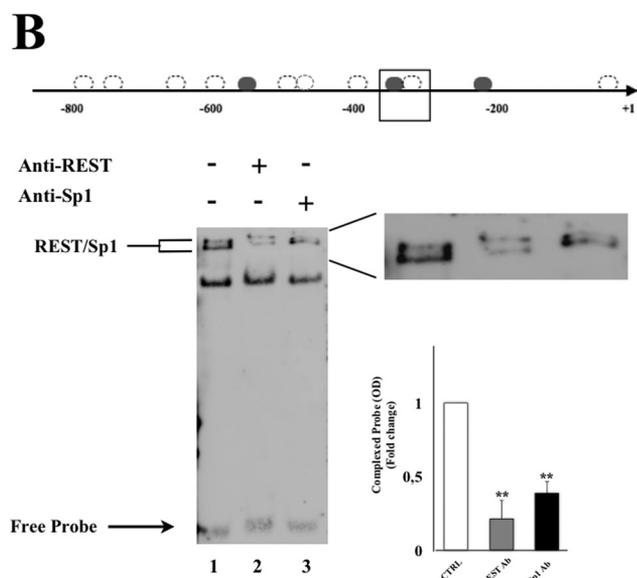
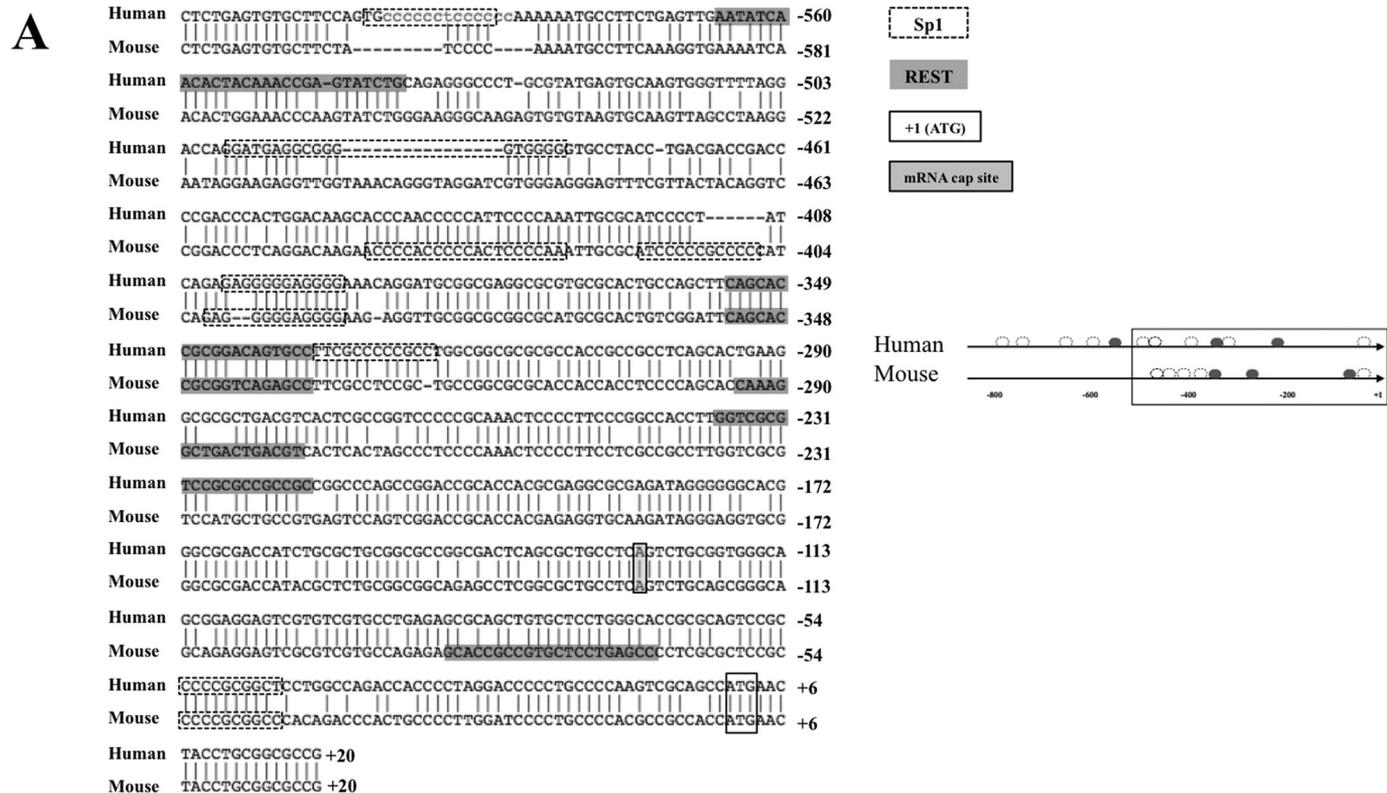
MouseSyn1-RV (HindIII), 5'-AGA AGC TTC CGC AGG TAG TTC ATG GTG-3'. All constructs were verified by DNA sequencing. Reporter and expression vectors were transiently co-transfected into cultured cells using Lipofectamine 2000 (Invitrogen). Control samples were co-transfected with the empty vector corresponding to the effector plasmids. pRL-TK-SV40 control plasmid was used as internal control. Luciferase activity was assayed after 48 h by using the Dual-Luciferase reporter assay system (Promega).

SYN1 Promoter Mutagenesis—Sp1 *cis*-sites on murine and human SYN1 promoters were mutated by site-directed mutagenesis. To inhibit Sp1 binding, the three purines (C/G) constituting the “core” elements of the Sp1 binding sites (identified by the Genomatix MatInspector tool) were mutated to three pyrimidines (A/T). Mutated residues are underlined. 50 μ g of wild type pGL3-Syn1m or pGL3-Syn1h promoter were PCR-amplified using *Pfu* DNA polymerase (Promega). The following primers were used: M1-FW, 5'-GGA CAA GAA CAT AAC CCC CAC TC-3'; M1-RV, 5'-GAG TGG GGG TTA TGT TCT TGT CC-3'; M2-FW, 5'-CAC CCC CAC TCT ATA AAT TGC GCA TC-3'; M2-RV, 5'-GAT GCG CAA TTT ATA GAG TGG GGG TG-3'; M3-FW, 5'-GCG CAT CCC TAA CCC CCA TCA GAG-3'; M3-RV, 5'-CTC TGA TGG GGG TTA GGG ATG CGC-3'; M4-FW, 5'-CCC CCA TCA GAG GAA TAG GGG AAG AGG TTG C-3'; M4-RV, 5'-GCA ACC TCT TCC CCT ATT CCT CTG ATG GGG G-3'; H1-FW, 5'-CCA GGA TGA ATT GGG GTG GGG GTG-3'; H1-RV, 5'-CAC CCC CAC CCC AAT TCA TCC TGG-3'; H2-FW, 5'-CAG GAT GAG GCG AAT TGG GGG TGC C-3'; H2-RV, 5'-GGC ACC CCC AAT TCG CCT CAT CCT G-3'; H3-FW, 5'-CTA TCA GAG AG GTA AAG GGG AAA CAG-3'; H3-RV, 5'-CTG TTT CCC CTT TAC CTC TCT GAT AG-3'; H4-FW, 5'-CAG TGC CTT CAA TCC CGC CTG G-3'; H4-RV, 5'-CCA GGC GGG ATT GAA GGC ACT G-3'. PCR conditions were: 95 °C, 5 min; 95 °C, 30 s; 55 °C, 30 s; 72 °C, 13 min. PCR products were digested using the DpnI enzyme (Promega) and transformed into DH5 α cells. Positive colonies were verified by DNA sequencing.

RNA Preparation and qRT-PCR—Total cellular RNA was prepared by using the RNeasy Plus mini kit (Qiagen), and isolated RNA was subjected to DNase I (Invitrogen) treatment. cDNA was synthesized starting from 1 μ g of total RNA according to the ImPromII reverse transcription kit (Promega) manual and used for qRT-PCR. The following primers were used: REST-FW, 5'-TTC ACA TTT ATA CGG GCG TTC-3'; REST-RV, 5'-CCT GCA GCA AGT GCA ACT AC-3'; Sp1-FW, 5'-TGG GTA CTT CAG GGA TCC AG-3'; Sp1-RV, 5'-TCC TTC TCC ACC TGC TGT CT-3'; Syn1-FW, 5'-AGC TCA ACA AAT CCC AGT CTC T-3'; Syn1-RV, 5'-CGG ATG GTC TCA GCT TTC AC-3'; HPRT1-FW, 5'-TCA GTC AAC GGG GGA CAT AAA-3'; HPRT1-RV, 5'-GGG GCT GTA CTG CTT AAC CAG-3'; GAPDH-FW, 5'-AGG TCG GTG TGA ACG GAT TTG-3'; GAPDH-RV, 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'; RPS9-FW, 5'-CTG GAC GAG GGC AAG ATG AAG C-3'; RPS9-RV, 5'-TGA CGT TGG CGG ATG AGC ACA-3'.

Immunofluorescence—Cells were fixed with 4% paraformaldehyde, 20% sucrose in PBS for 15 min at room temperature

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and permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Samples were blocked for 30 min in Immunofluorescence Buffer (2% BSA, 10% goat serum in PBS). Primary and secondary antibodies were diluted in Immunofluorescence Buffer and incubated for 45 min at room temperature. Coverslips were mounted in Mowiol 4-88.

Bisulfite DNA Analysis—Genomic DNA was extracted from various tissues by using the DNeasy blood and tissue kit (Qiagen). DNA (2 μ g) was digested using XhoI restriction enzyme. Treated DNA was purified by using the PCR purification kit (Qiagen), eluted in 30 μ l of H₂O, and denatured for 20 min at 90 °C. After incubation on ice for 1 min, 7.5 μ l of 3 M NaOH were added, and samples were incubated 20 min at 45 °C. After the addition of 18 μ l of 0.1 M hydroquinone and 312 μ l of 2 M sodium bisulfite, pH 5.0, samples were incubated overnight at 55 °C. Treated DNA was purified using the PCR purification kit (Qiagen), and 50 μ l were desulfonated by incubation with 5 μ l of 3 M NaOH, 15 min at 40 °C. DNA was purified and used as template in PCR reactions. Bisulfite-treated mouse *SYN1* promoter was amplified using the following primers: MouseBis-FW, 5'-TTT ATG TTT TTT TTT GAG TGT GT-3'; MouseBis-RV, 5'-CCC TCC CCC TCT AAT AAA AA-3'. Amplified DNA was subjected to direct sequencing.

Statistical Analysis—Results are expressed as means \pm S.E. throughout. Data were analyzed by either the unpaired Student's *t* test or one-way ANOVA followed by Tukey's multiple comparison test.

RESULTS

Sp1 Binds the *SYN1* Promoter in Proximity to REST cis-Sites—The 5'-flanking region of the *SYN1* gene is highly conserved across species (16, 28). In particular, human and mouse *SYN1* gene promoters show a high degree of homology (82%) in the first 600 bp upstream of ATG (Fig. 1A), a GC-rich region previously identified as fundamental for gene expression in neural cells (16). To further address the molecular mechanisms determining *SYN1* promoter activation, we subjected human and mouse sequences to computational prediction of transcriptional factor *cis*-sites using the Genomatix MatInspector database (29). We identified several binding sites for the ubiquitous transcriptional factor Sp1 dispersed within the previously characterized RE1 elements that act as binding sites for the transcriptional repressor REST (Ref. 17, Fig. 1A). Because Sp1 has been associated with the activation of target genes (30, 31), we hypothesized a direct role of this protein in *SYN1* gene expression.

To assess whether Sp1 physically interacts with the *SYN1* promoter, we performed EMSAs using representative regions of human and mouse *SYN1* promoters, containing

both Sp1 and REST *cis*-sites, as probes (Fig. 1, B and C, top). When probes were incubated with nuclear extracts from human SH-SY5Y or murine N2a cells, the formation of slower migrating DNA-protein complexes was observed (Fig. 1, B and C, lane 1). The presence of Sp1 was revealed through supershift competition (Fig. 1, B and C, lane 3). Interestingly, the Sp1-containing complex was co-supershifted using a specific antibody against REST (Fig. 1, B and C, lane 2). The co-supershift was likely due to an interaction between the two factors, as described previously (32). Moreover, to prove a direct association of Sp1 with the *SYN1* promoter also in the nuclear environment, we performed ChIP assays on extracts from SH-SY5Y or N2a cells. As shown in Fig. 1, D and E, when chromatin was precipitated using anti-Sp1 antibodies, we were able to amplify specific *SYN1* promoter regions in both human and mouse samples. Altogether, these results demonstrate that Sp1 is able to interact with both human and mouse *SYN1* promoters *in vitro* and in live cells, in close proximity to the previously reported REST *cis*-sites.

Sp1 and REST Interplay on the *SYN1* Promoter—Because Sp1 can bind both human and mouse *SYN1* promoters close to REST binding sites, we asked whether there is a functional interaction of these transcriptional factors on this promoter region. Human and mouse *SYN1* promoter sequences were cloned in the pGL3 reporter vector and transiently co-transfected in SH-SY5Y and N2a cells, respectively. When Sp1 expression was down-regulated using a specific siRNA, the transcriptional activity of the *SYN1* promoter was significantly inhibited, whereas the opposite effect was achieved by overexpressing exogenous Sp1 (Fig. 2, A and B, lanes 2 and 4). When the endogenous REST content was reduced using a specific shRNA, an increase of *SYN1* promoter activity was observed. Moreover, an additive increase occurred when REST down-regulation was coupled to Sp1 overexpression (Fig. 2, A and B, lanes 5 and 6). On the other hand, the repression of *SYN1* activity caused by overexpression of exogenous REST was not rescued by Sp1 overexpression (Fig. 2, A and B, lanes 8 and 9). Because the transcriptional factors Sp1 and Sp3 exhibit a very similar DNA binding specificity (25), we also evaluated Sp3 activity on *SYN1* promoter. Interestingly, when Sp3 was overexpressed, a reduction in the activation of both mouse and human *SYN1* promoters was observed (Fig. 2, A and B, lanes 10 and 11). Taken together, these results show that Sp1 can directly activate both human and mouse *SYN1* gene promoters and that REST can impair *SYN1* transcription by inhibiting Sp1 positive activity.

FIGURE 1. Sp1 binds human and mouse *SYN1* promoters. A, left, alignment of the human (Gene ID: 6853) and mouse (Gene ID: 20964) *SYN1* gene promoter sequences, from -560 to +20. Right, schematic representation of the human and mouse *SYN1* promoters. Sp1 (open circles) and REST (closed circles) *cis*-sites are highlighted. B and C, gel shift assays of biotinylated fragments of human (B) and mouse (C) *SYN1* promoters. The regions used for the assay are boxed in the top panel (human, -371 to -295; mouse -428 to -319). The biotinylated probes were incubated with nuclear extracts from SH-SY5Y or N2a cells. Specific REST and Sp1 signals were identified by supershift assays by incubating the extracts with antibodies (Ab) against REST or Sp1, as indicated, before the addition of the probe. The insets show higher magnification images of the REST/Sp1 bands. Quantification of the complexed probes, expressed as percentage of control (CTRL), is provided. Arrows indicate free probe (*, $p < 0.05$; **, $p < 0.01$; one-way ANOVA followed by Tukey's multiple comparison test; $n = 3$ independent experiments). D and E, chromatin immunoprecipitation of human (D) and mouse (E) *SYN1* promoters was performed from SH-SY5Y and N2a cells, respectively. The PCR-amplified regions are boxed in the top panel (human, -780 to -280; mouse -520 to -256). Sp1 binding was revealed using 2 μ g of anti-Sp1 antibody. No antibodies (No Ab) or rabbit IgGs (IgG) were used as negative controls.

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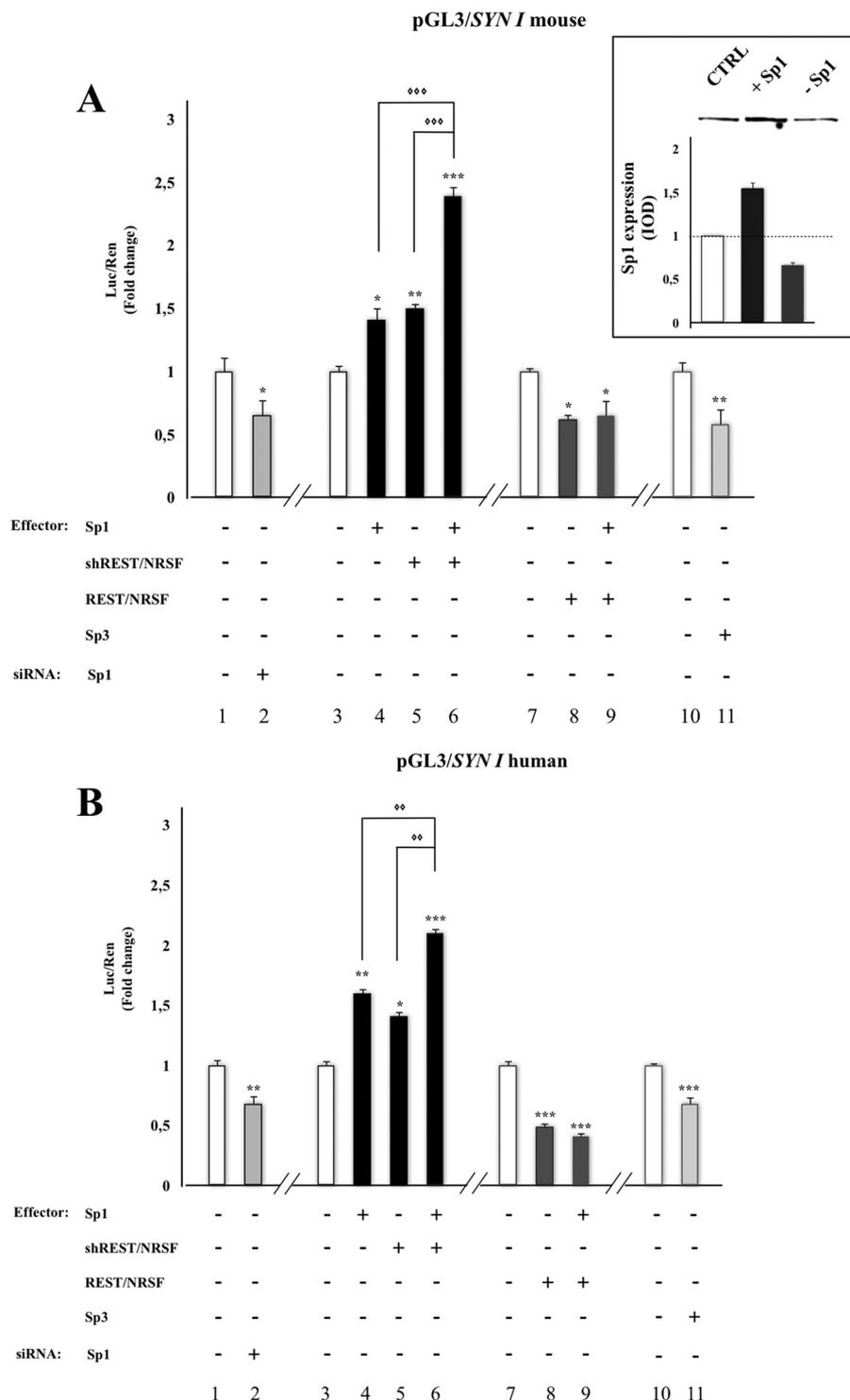


FIGURE 2. Sp1 and REST interplay on the SYN1 promoter. A and B, N2a (A) and SH-SY5Y (B) cells were transfected with a vector containing either the human or the murine *SYN1* promoter as reporter gene in the absence (white bars) or presence (black/gray bars) of expression plasmids for Sp1, Sp3, REST, shRNA against REST, and siRNA against Sp1, as indicated. Control samples were co-transfected with the empty vector corresponding to the effector plasmids or with scramble siRNA. Luciferase activity was measured 48 h after transfection. Data were first normalized to the activity of the co-transfected TK-*Renilla* reporter vector and subsequently to the activity of the reporter gene alone, set to 1 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, one-way ANOVA followed by Tukey's multiple comparison test, * versus respective control; ◇ versus the indicated group; $n = 3-6$ independent experiments). *Luc/Ren*, luciferase/Renilla ratio. *Inset* in A, expression of Sp1 protein in the presence of either Sp1 vector or siRNA against Sp1. *IOD*, Integrated Optical Density. A representative immunoblot and densitometric analysis show the changes in Sp1 immunoreactive levels upon overexpression (+Sp1; black bar) and siRNA transfection (-Sp1; gray bar) when compared with control samples (CTRL; white bar).

A Conserved Sp1 cis-Site Is Required for Efficient SYN1 Promoter Activation—To understand which of the putative Sp1 binding sites are the major players in the modulation of Syn I expression, we performed site-directed mutagenesis of Sp1 *cis*-

sites on the mouse and human *SYN1* promoters (see “Experimental Procedures”). In particular, we restricted our attention to the genetic region within the nucleotides -200 and -550, comprising four Sp1 *cis*-sites whose constitutive *trans*-activity

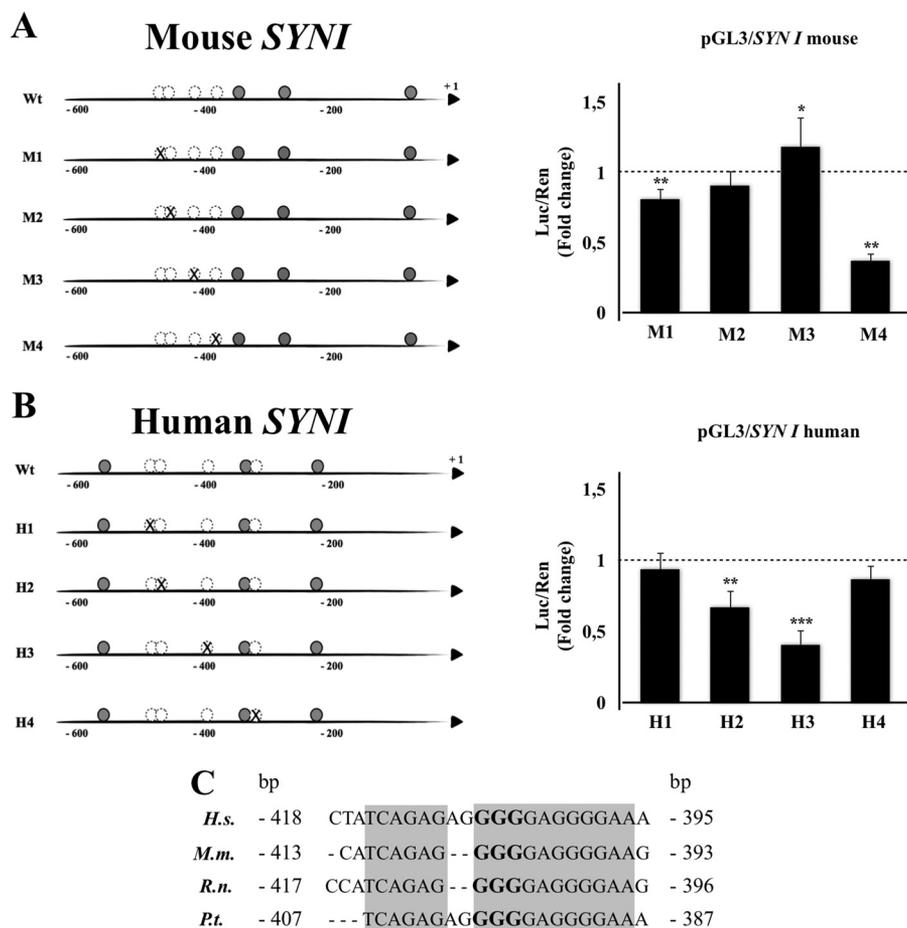


FIGURE 3. A conserved Sp1 cis-site is required for an efficient *SYN1* promoter activation. *A* and *B*, left, schematic representation of the mouse/human *SYN1* promoter constructs. Four predicted Sp1 cis-sites (M1–M4/H1–H4) were mutated in the mouse/human *SYN1* promoter region by site-directed mutagenesis to inhibit Sp1 binding. Sp1 (open circles) and REST (closed circles) cis-sites are highlighted; mutated sites are crossed. Right, wild type and mutant constructs were transiently transfected into N2a/SHSY5Y cells, and luciferase activity was measured. Data were first normalized to TK-*Renilla* activity and subsequently normalized to the activity of the wild type promoter, set to 1 (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, Student's *t* test versus wild type promoter, $n = 5$ independent experiments). *Luc/Ren*, luciferase/*Renilla* ratio. *C*, sequence comparison of the conserved purine-rich motif corresponding to the M4 (mouse) and H3 (human) Sp1 cis-sites on the *SYN1* promoter. *H.s.*, *Homo sapiens*; *M.m.*, *Mus musculus*; *R.n.*, *Rattus norvegicus* (Gene ID: 24949); *P.t.*, *Pan troglodytes* (Gene ID: 736611). Sp1 core nucleotides are in bold. bp numbers indicate the distances from the respective ATG.

has been previously characterized (33) (Fig. 3, *A* and *B*, left). By performing luciferase assays on the various mouse *SYN1* promoter mutants, we reported a decrease of activity when Sp1 binding was inhibited at the sites referred to as M1 (–441 to –454) and M4 (–393 to –407) when compared with the wild type construct. Conversely, an increase of murine *SYN1* activity was observed when the mutation affected the cis-site M3 (–413 to –425) (Fig. 3*A*, right). A similar analysis of the human *SYN1* promoter revealed that mutations of the H2 (–488 to –503) and H3 (–395 to –410) cis-sites strongly impaired *trans*-activity when compared with the wild type construct (Fig. 3*B*, right). Interestingly, the mutations that affected *SYN1* promoter activity to the greatest extent, *i.e.* M4 and H3, are homologous in mouse and human. Moreover, a comparative analysis revealed that this Sp1 site is the most conserved across species (Fig. 3*C*). Together, these data strongly support the idea that Sp1 can positively drive *SYN1* gene transcription, acting on a conserved promoter region.

Differential Expression of Sp1, REST, and Syn I during Neuronal Differentiation—Several studies have shown an inverse correlation between REST expression and *SYN1* transcription

during neural development and network maturation (33, 34). To follow Sp1 expression during neuronal differentiation, we treated N2a cells with RA and monitored variations of Sp1, REST, and Syn I mRNA levels by qRT-PCR. Following RA treatment, N2a cells assumed a neuron-like morphology (Fig. 4*A*). By comparing gene expression in undifferentiated and differentiated cells, we observed a decrease in REST and an increase in Syn I mRNA levels, in accordance with previous results (35), whereas no changes in Sp1 transcription were revealed (Fig. 4*B*). The higher *SYN1* promoter activity after RA-induced N2a cell differentiation was further confirmed by luciferase activity assays, suggesting that the increase in Syn I mRNA was not due to post-transcriptional events affecting mRNA stability (Fig. 4*C*). By using the same approach, we monitored REST, Syn I, and Sp1 expression in primary cultures of mouse cortical neurons at 1, 4, and 10 days *in vitro* (DIV) (Fig. 4*D*). Similar to that observed in N2a cells, the progressive increase of Syn I expression during neuronal network formation was paralleled by a reduction of REST mRNA levels. Interestingly, the Sp1 transcriptional rate, constant at early stages *in vitro*, increased in older cultures (DIV 10; Fig. 4*E*). These results suggest that a

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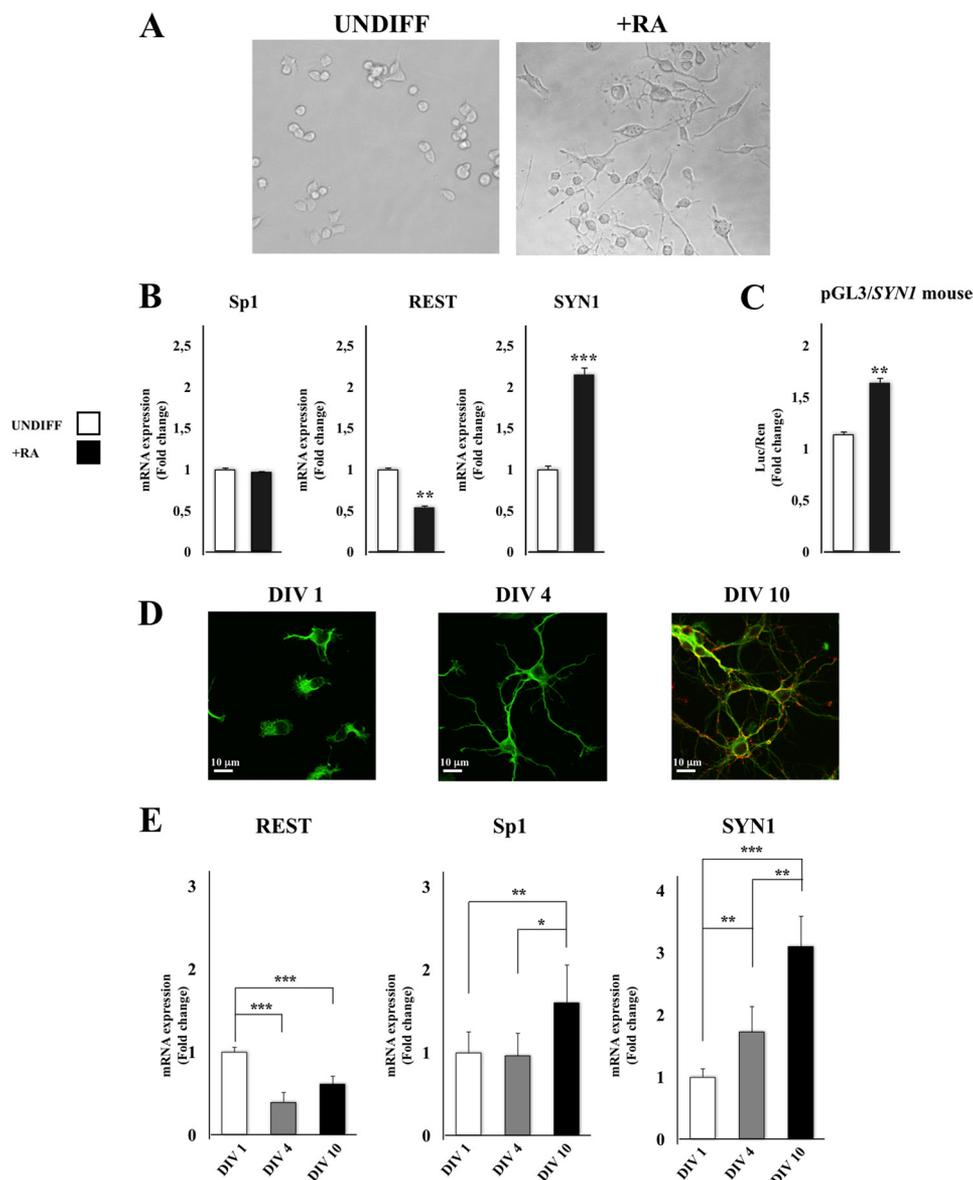


FIGURE 4. Differential expression of Sp1, REST, and Syn I during neuronal differentiation. *A*, representative images of undifferentiated (UNDIFF) and RA-differentiated (+RA) N2a cells. *B*, Sp1, REST and Syn I mRNA levels were quantified by qRT-PCR in undifferentiated (white bars) and differentiated (black bars) cells. RPS9, GAPDH and HPRT1 were used as control housekeeping genes. *C*, a reporter vector containing the mouse SYN1 promoter (pGL3/SYN1m) was transfected in N2a cells, and luciferase activity was measured in untreated or RA-treated cells. Data were normalized to TK-Renilla activity and subsequently normalized to the activity under undifferentiated conditions, set to 1 (B, C: **, $p < 0.01$; ***, $p < 0.001$, Student's *t* test; $n = 3$ independent experiments). Luc/Ren, luciferase/Renilla ratio. *D*, representative images of primary cortical neurons at various DIV, stained for a neuron-specific cytoskeletal protein (β III tubulin, green channel). DIV 10 cultures were co-stained for Syn I (red channel), evidencing the synaptic localization of the Syn I protein in mature cultures. Scale bars, 10 μ m. *E*, REST, Sp1, and Syn I mRNA levels were quantified by qRT-PCR at various DIV (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus the indicated group; one-way ANOVA followed by Tukey's multiple comparison test; $n = 6$ independent experiments, from 6 separate litters).

gradual decrease of REST nuclear levels allows a constitutive Sp1 transcriptional activity on the SYN1 promoter during neuronal differentiation.

REST Affects Sp1 Stability on the SYN1 Promoter—To further investigate the molecular mechanisms underlying the functional interplay between REST and Sp1 on the SYN1 promoter, we performed ChIP assays in N2a cells and evaluated Sp1 binding to the SYN1 promoter (−490 to −246) before and after differentiation (Fig. 5A). We found that the amount of Sp1 bound to the SYN1 promoter increased in differentiated cells when compared with undifferentiated conditions. A similar result was obtained in human SH-SY5Y cells (data not shown).

To verify whether the amount of Sp1 bound to the promoter was related to the amount of REST present in the cell, we performed ChIP from N2a cells, under conditions in which the amount of endogenous REST was either decreased by specific shRNA or increased by overexpression. Although no change in Sp1 binding to the SYN1 promoter was observed upon REST overexpression, a significant increase in Sp1 binding was reported after depletion of REST (Fig. 5B). qRT-PCR analysis confirmed that Sp1 transcription was not affected by alterations of REST levels, whereas, as expected, SYN1 expression was inversely correlated to the amount of REST (Fig. 5C). Altogether, these data show that Sp1 binding to the SYN1 promoter

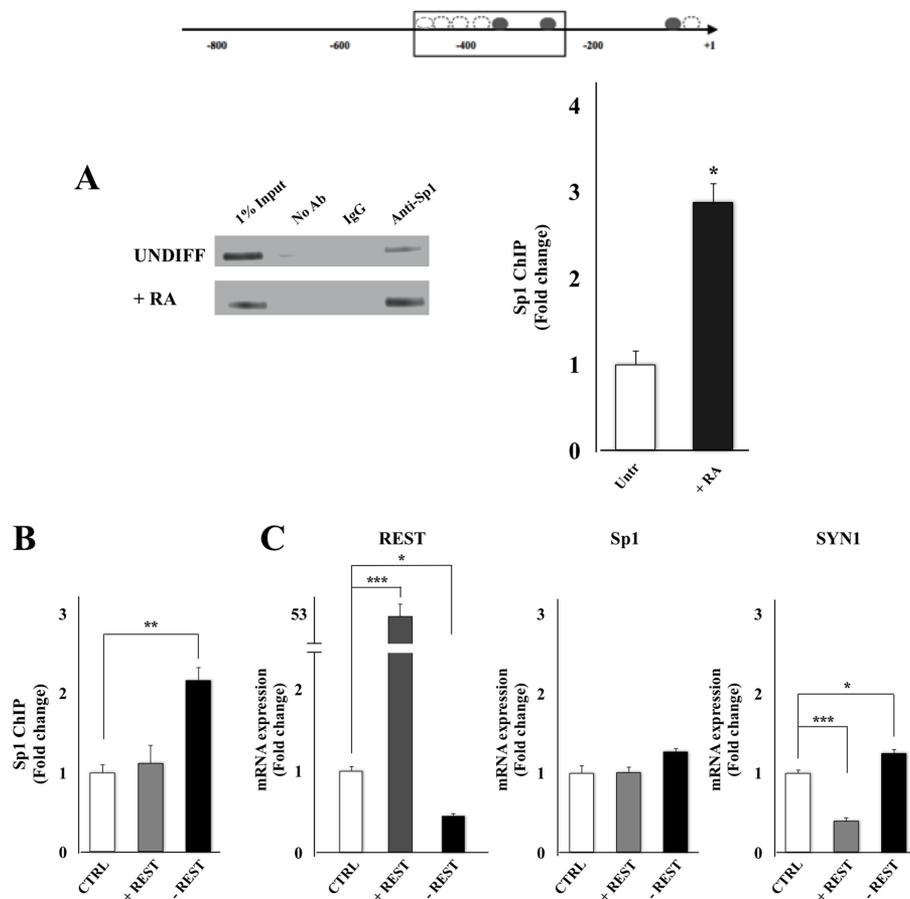


FIGURE 5. REST affects Sp1 stability on the SYN1 promoter. A, chromatin immunoprecipitation of the murine SYN1 promoter region (–520 to –256, boxed region in the top panel) was performed in undifferentiated (UNDIFF) and RA-differentiated (+RA) N2a cells using 2 μ g of anti-Sp1 antibodies. A representative image of the gel is shown on the left, whereas the quantification is shown on the right (*, $p < 0.05$, Student's t test; $n = 3$ independent experiments). No Ab, no antibody; IgG, rabbit IgG; Untr, untreated. B, N2a cells were transfected with shRNA against REST (–REST) or with a REST expression vector (+REST) and then subjected to chromatin immunoprecipitation of the SYN1 promoter region (–520 to –256), using 2 μ g of anti-Sp1 antibodies. Sp1 binding was normalized against the input value and subsequently normalized to the binding in the control (CTRL) sample, set to 1. C, variations of REST, Sp1 and Syn I mRNA under the conditions described in B were evaluated by RT-PCR (B and C, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ versus control, one-way ANOVA followed by Tukey's multiple comparison test; $n = 3$ separate experiments).

is fine-tuned by REST and that the reduction of REST nuclear levels allows the binding of Sp1, resulting in an increase of Syn I expression.

Methylation Inhibits Syn I Expression through Sp1 Displacement—CpG-rich DNA sequences are susceptible to methylation, leading to gene silencing (36). Computational analysis of the human and mouse SYN1 5'-flanking regions with the MethPrimer program (37) identified an extended CpG island in both sequences, from –420 to –30 (human and mouse sequences contain 43 and 55 CpG, respectively; Fig. 6A). It has been reported that cytosine methylation may inhibit Sp1 binding to its consensus sites, therefore affecting its positive *trans*-activity (38, 39). Thus, we asked whether this epigenetic modification might play a role in the modulation of Sp1 binding to the SYN1 promoter. To answer this question, we analyzed the methylation status of the promoter region (–470 to –390) surrounding the identified Sp1 *cis*-sites in tissues and cells expressing or not expressing Syn I. To exclude methylation deriving from X chromosome inactivation, we analyzed only samples coming from male mice or male-derived cell lines. Genomic DNA was treated with bisulfite and then amplified by PCR (Fig. 6B), whereas Syn I protein expression was confirmed

by Western blot analysis (Fig. 6C). Upon bisulfite treatment, all unmethylated cytosines are converted to uracils, which are PCR-amplified as thymidines, whereas the methylated cytosines are not modified. As shown in Fig. 6B, the SYN1 promoter was completely unmethylated in mouse cortex and hippocampus, which express high levels of Syn I. On the other hand, DNA from non-neuronal RAW 264.7 cells or from neuronal precursors, which do not express Syn I, showed a specific methylation pattern (arrows) overlapping with, or in close proximity to, Sp1 binding sites. However, tissues such as heart and kidney, which also do not express Syn I, did not show any methylation of the SYN1 promoter, suggesting the presence of other tissue-specific repressing mechanisms, such as the previously identified histone deacetylation (40).

We further investigated the role of methylation in controlling SYN1 gene expression using the demethylating agent 5-azacytidine (5-AzaC). Treatment with 5-AzaC was able to induce SYN1 transcription in RAW 264.7 cells (Fig. 7A), without affecting the endogenous expression of Sp1 (not shown). When we performed ChIP experiments on methylated and 5-AzaC-treated DNA, we found that both Sp1 and REST displayed an increased binding affinity to the unmethylated SYN1

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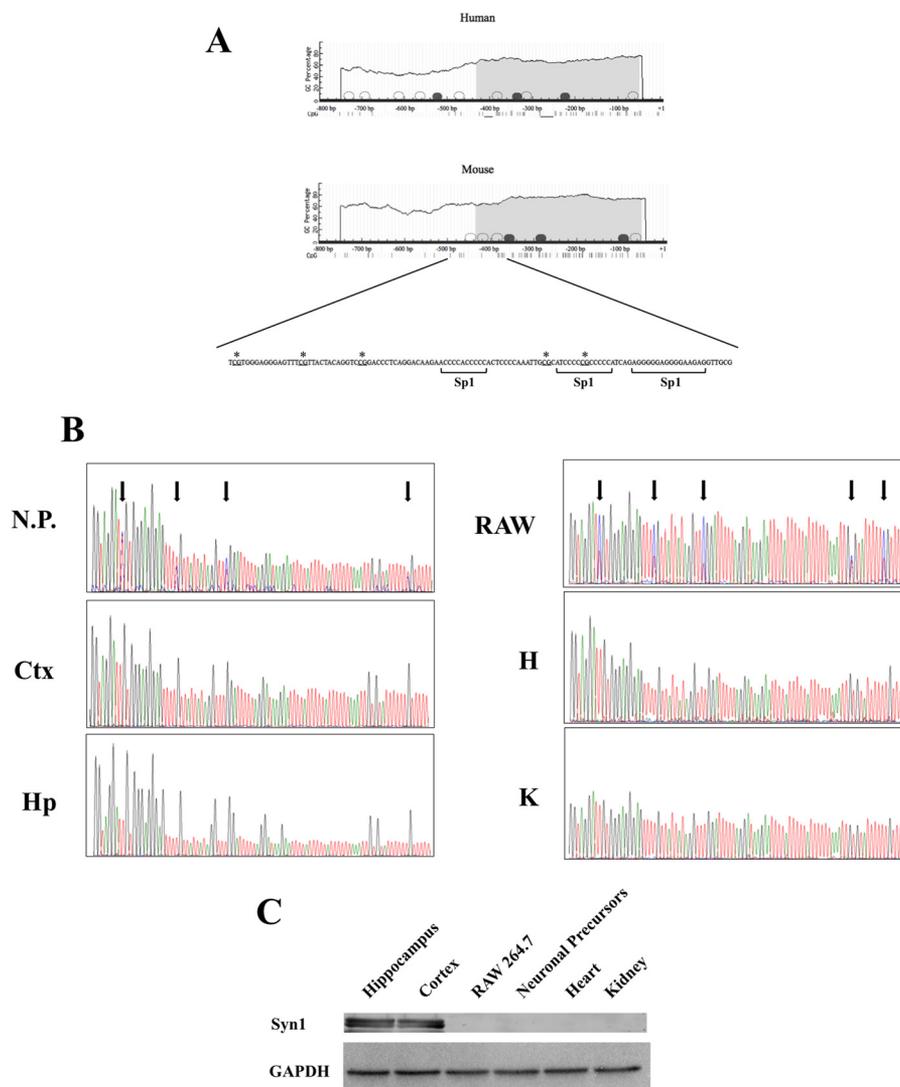


FIGURE 6. Differential methylation pattern of the SYN1 promoter. *A*, predicted CpG islands (gray areas) in human and mouse SYN1 promoters. Sp1 (open circles) and REST (closed circles) cis-sites are highlighted. *B*, sequences of murine SYN1 promoter from bisulfite-treated Syn I-expressing (mouse hippocampus and cortex) and -nonexpressing (RAW 264.7 macrophage cells, neuronal precursors, heart, and kidney) samples revealed a cell type-specific pattern of methylation. Arrows indicate methylated residues. *N.P.*, neuronal precursors; *Ctx*, cortex; *Hp*, hippocampus; *RAW*, RAW 264.7; *H*, heart; *K*, kidney. *C*, Western blot analysis of the same samples as in *B* to detect Syn I expression. GAPDH immunoreactivity was used as a control for equal loading.

promoter (Fig. 7*B*). As discussed later, it is likely that the binding of REST to the unmethylated SYN1 promoter is ineffective to repress SYN1 transcription due to the inability of the repressor complex to form on 5-AzaC-treated, unmethylated DNA. Thus, CpG methylation in proximity to Sp1 cis-sites contributes to the modulation of murine SYN1 promoter region by affecting the binding of positive transcription factors, and therefore may play an important role in the regulation of Syn I expression.

DISCUSSION

Multicellular organisms are able to modulate gene expression through combinatorial interactions between transcriptional factors and epigenetic modifications. Syn I is a neuron-specific protein that plays fundamental roles during nervous system development and maturation (9). SYN1 expression depends on a conserved sequence of 600 bp upstream of the transcriptional start site, containing cis-sites for the transcriptional repressor REST. Previous studies identified in this region

functional consensus motifs for the cAMP-responsive element-binding protein (CREB) (41) and the zif268/egr-1 factor (42), whose action was shown to be strictly dependent on elevated cAMP levels (43).

In this study, we focused on the molecular mechanisms that allow the constitutive transcription of the SYN1 gene in neuronal cells. By subjecting the SYN1 promoter region to computational analysis, we predicted various cis-sites for the ubiquitous transcriptional factor Sp1, whose activity has been previously linked to the activation of tissue-specific TATA-less promoters (25, 30, 31). We subsequently demonstrated that indeed Sp1 binds to both human and mouse SYN1 promoters. Interestingly, the majority of Sp1 binding sites are in close proximity to the previously characterized REST cis-sites, suggesting the interplay between these two factors to modulate SYN1 transcription. The physical association between Sp1 and REST has been previously reported for the ITPKA, Bsx, and synaptophysin I genes (44–46), where REST exerts an inhibitory role on

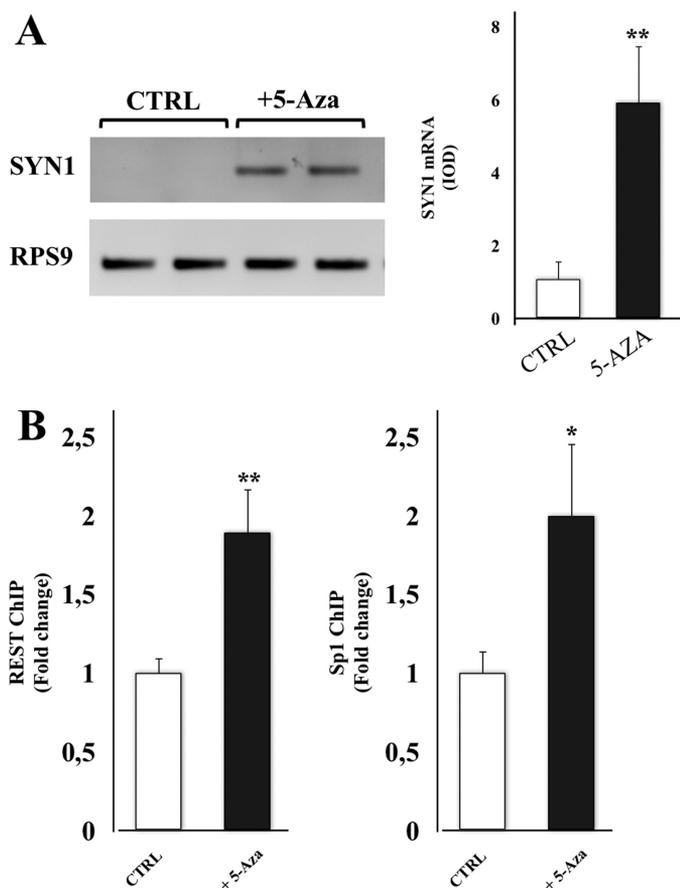


FIGURE 7. Demethylation of *SYN1* promoter induces *SYN1* transcription. *A*, left, representative *SYN1* RT-PCR from RAW 264.7 cells under control condition (CTRL) or upon 5-azacytidine (5-Aza) treatment. The constitutive *RPS9* gene was amplified as a control. The corresponding quantification is presented on the right. IOD, Integrated Optical Density. *B*, chromatin immunoprecipitation of the murine *SYN1* promoter region (−520 to −256) was performed in untreated (CTRL) or 5-AzaC-treated RAW 264.7 cells using 2 μ g of anti-Sp1 or anti-REST antibodies. Sp1 and REST binding was normalized to the input value and subsequently normalized to the binding in the control samples, set to 1 (*, $p < 0.05$, **, $p < 0.01$; Student's *t* test; $n = 3$ independent experiments).

Sp1 transcriptional activity (32). Our functional data support a similar role for REST in the modulation of Sp1 constitutive activity on the *SYN1* promoter. Moreover, we identified a highly conserved purine-rich region on the *SYN1* promoter (M4/H3) as fundamental for a full activation of gene transcription.

Given the very similar DNA binding specificity of the transcriptional factors Sp1 and Sp3 (25), we also evaluated whether Sp3 has any modulatory activity on the *SYN1* promoter. Opposite to Sp1, a reduction in the activation of *SYN1* promoter was observed when Sp3 was overexpressed. These results are in line with previous findings, demonstrating that Sp3 can repress Sp1-mediated activation of promoters containing two or more Sp1 *cis*-sites (25, 47), such as the *SYN1* promoter. The increased mouse *SYN1* promoter activity observed in the M3 mutant suggests that M3 could be a preferential binding site for Sp3.

Syn I expression is dynamically regulated during development and increases during neuronal differentiation and synapse formation (10). By using RA-treated N2a cells and primary cultures of mouse cortical neurons as a model of neuronal dif-

ferentiation, we observed that the increase in *SYN1* transcription was paralleled by a decrease in REST, resulting in an increased binding affinity of Sp1 on the *SYN1* promoter. On the other hand, Sp1 expression was constant at early stages *in vitro* and increased in mature cultures.

The *SYN1* promoter comprises a CpG-rich region (−420 to −30), where the Sp1 binding sites are located. Several studies have demonstrated that CpG methylation of gene promoters is important in the regulation of cell type-specific expression (48). In particular, DNA methylation interferes with Sp1 binding to target GC boxes (49–52). We identified a specific pattern of methylation of the *SYN1* promoter in close proximity to, or overlapping with, Sp1 sites in undifferentiated neural precursors as well as in non-neural macrophage-derived RAW 264.7 cells, which do not express Syn I. On the contrary, the promoter was completely unmethylated in mouse brain cortex and hippocampus, which express high levels of Syn I protein. However, in samples from non-neuronal tissues, such as heart and kidney, Sp1 sites were unmethylated in the absence of Syn I expression, thus suggesting that methylation may affect *SYN1* expression in a cell- and tissue-specific fashion.

The inhibitory role of methylation in the transcriptional activation of *SYN1* was demonstrated by the ability of 5-azacytidine, a demethylating drug, to restore Syn I expression in RAW 264.7 cells. However, our ChIP experiments indicate that not only Sp1, but also REST, binds with higher affinity to unmethylated DNA. This apparent paradox was already described for other REST-regulated genes, such as *NaCh II*, *Gad I*, and *M4*. Demethylation of these regions resulted in the release of methyl CpG binding domain (MBD) proteins, such as MeCP2 and CoREST1, whereas REST association was not affected by the methylation status (53). These data suggest that REST bound to unmethylated DNA is ineffective to repress *SYN1* transcription due to the lack of other members of the repressor complex. Thus, differential CpG methylation may represent a novel mechanism to regulate Syn I expression by modulating the binding and/or the activity of transcriptional regulators to the *SYN1* promoter region.

In conclusion, our experiments introduce Sp1 as a key player in the constitutive neuron-specific expression of the *SYN1* gene. Syn I expression is the result of the functional interplay between REST and Sp1, with REST exerting an inhibitory action on Sp1 transcriptional activity. Moreover, *SYN1* promoter is subjected to differential methylation, which may contribute to determine the specific pattern of Syn I expression. On the basis of our data, we propose a model whereby repression of *SYN1* transcription could be achieved in two different ways: (i) Sp1 binding to the *SYN1* promoter is constitutively blocked by a negative chromatin environment induced by the REST repressor complex (Fig. 8A); and (ii) methylation may play an additional role in the silencing of *SYN1* transcription by recruiting MBD proteins, which cooperate with the REST complex to mediate gene silencing (21, 53), and preventing Sp1 binding (Fig. 8B). When REST levels decrease, such as during neuronal differentiation, chromatin repression is relieved, allowing Sp1 binding and full activation of *SYN1* transcription. In the second scenario, removal of methylation frees GC boxes and enhances Sp1 binding to *cis*-sites. Therefore, DNA methylation could

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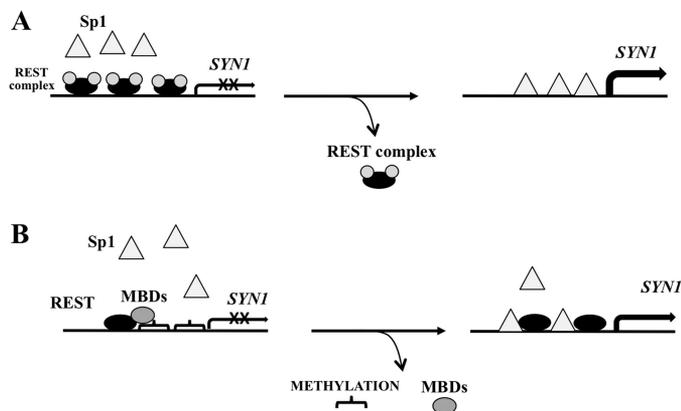


FIGURE 8. Models of Sp1-mediated SYN1 promoter activation. A, Sp1 binding to the SYN1 promoter is constitutively blocked by a negative chromatin environment induced by the REST repressor complex. When REST levels decrease, such as during neuronal differentiation, chromatin repression is relieved, allowing Sp1 binding and full activation of SYN1 transcription. B, at very precocious stages of neuronal differentiation or in some non-neuronal cells, methylation may play an additional role in the silencing of SYN1 transcription by recruiting MBD proteins on methylated CpGs and preventing Sp1 binding. Removal of methylation frees GC boxes and enhances Sp1 binding to cis-sites. At the same time, REST-mediated gene repression is lowered, further enhancing SYN1 transcription.

represent an additional epigenetic mechanism, in addition to the previously characterized histone deacetylation and/or H3K9 methylation (40), to induce gene repression by impairing Sp1 binding to the SYN1 promoter.

The elucidation of the mechanisms underlying basal activation of neuron-specific genes is fundamental to improve our understanding of brain pathologies, such as epilepsy, autism, dementia, or major depressive disorders in which the transcription pattern is often dysregulated (7, 8). Besides tissue-specific methylation, recent findings have highlighted a regulatory role of DNA methyltransferases in the modulation of synaptic plasticity or in the onset of brain diseases by controlling gene expression in mature neurons (54–56). We hypothesize that methylation could also serve as a transient modulator of transcription to down-regulate gene expression without histone methylation/deacetylation. Future experiments will aim at better characterizing SYN1 promoter epigenetic marks under physiological and pathological conditions.

Acknowledgments—We thank Prof. J. Meldolesi (Dipartimento di Biotecnologie, San Raffaele Scientific Institute, Milan, Italy) for the kind gift of the REST and shREST constructs, Dr. Andrea Contestabile for providing neuronal precursor cells, Dr. Marina Nanni and Claudia Chiabrera (Istituto Italiano di Tecnologia (IIT), Genova, Italy) for valuable help with cell cultures and Dr. Ennio Albanesi (IIT, Genova, Italy) for help with genetic sequencing.

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