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Yin Yang 1 Is a Repressor of Glutamate Transporter EAAT2, and It Mediates Manganese-Induced Decrease of EAAT2 Expression in Astrocytes

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Impairment of astrocytic glutamate transporter (GLT-1; EAAT2) function is associated with multiple neurodegenerative diseases, including Parkinson's disease (PD) and manganism, the latter being induced by chronic exposure to high levels of manganese (Mn). Mn decreases EAAT2 promoter activity and mRNA and protein levels, but the molecular mechanism of Mn-induced EAAT2 repression at the transcriptional level has yet to be elucidated. We reveal that transcription factor Yin Yang 1 (YY1) is critical in repressing EAAT2 and mediates the effects of negative regulators, such as Mn and tumor necrosis factor alpha (TNF-α), on EAAT2. YY1 overexpression in astrocytes reduced EAAT2 promoter activity, while YY1 knockdown or mutation of the YY1 consensus site of the EAAT2 promoter increased its promoter activity and attenuated the Mn-induced repression of EAAT2. Mn increased YY1 promoter activity and mRNA and protein levels via NF-κB activation. This led to increased YY1 binding to the EAAT2 promoter region. Epigenetically, histone deacetylase (HDAC) classes I and II served as corepressors of YY1, and, accordingly, HDAC inhibitors increased EAAT2 promoter activity and reversed the Mn-induced repression of EAAT2 promoter activity. Taken together, our findings suggest that YY1, with HDACs as corepressors, is a critical negative transcriptional regulator of EAAT2 and mediates Mn-induced EAAT2 repression.

Glutamate is the main excitatory neurotransmitter in the central nervous system (CNS), and it plays a vital role in synaptic plasticity, learning, memory, and long-term neuronal potentiation (1). However, excessive extracellular glutamate levels cause hyperactivation of glutamate receptors, leading to excitotoxic cell death (2). Glutamate transporters are responsible for clearing glutamate from the synaptic clefts, thus maintaining its homeostasis. Glutamate transporter dysfunction has been linked to neurological disorders, including stroke, epilepsy, amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson disease (PD) (reviewed in reference 3). In humans, among the five subtypes of Na^+-dependent glutamate transporters (excitatory amino acid transporters EAAT1 to EAAT5), EAAT1 and EAAT2, homologs of glutamate/aspartate transporter (GLAST) and GLT-1 in rodents, are preferentially expressed in astrocytes and considered the major transporters, with EAAT2 alone accounting for >80% of synaptic glutamate clearance (3, 4). Since the dysregulation of EAAT2 is associated with various neurological disorders, understanding the regulatory mechanism of this transporter is critical for the development of therapeutics to mitigate glutamate-mediated pathologies (5).

Several positive and negative modulators of EAAT2 at the transcriptional level have been identified, but the negative regulatory mechanisms of EAAT2 have yet to be established. Treatment of primary human fetal astrocytes with epidermal growth factor (EGF), transforming growth factor α (TGF-α), and cyclic AMP analogs upregulates EAAT2 mRNA and protein levels via protein kinase A, phosphatidylinositol 3-kinase (PI3K), and NF-κB (6). Beta-lactam antibiotics stimulate EAAT2 expression, and, in particular, ceftriaxone exerts neuroprotective effects by increasing EAAT2 transcription via the NF-κB signaling pathway (7, 8). Our previous findings revealed that estrogen and selective estrogen receptor modulators (SERMs), such as tamoxifen, also increase glutamate transporter expression via the activation of NF-κB (9). On the other hand, one study reported that tumor necrosis factor alpha (TNF-α) decreased EAAT2 expression by activation of NF-κB upon N-myc recruitment (10).

Exposure to high manganese (Mn) levels induces manganism, a disease having pathological symptoms similar to those of PD (reviewed in reference 11). Astrocytes are the cellular target of Mn toxicity, which is primarily mediated by oxidative stress and impairment of glutamate transporter function (12, 13). Mn also alters glutamate/glutamine homeostasis by downregulating the expression and function of glutamine transporters, resulting in increased glutamate levels and ensuing excitotoxic injury (14). We along with others have shown that Mn impaired glutamate transporter function by decreasing GLT-1 mRNA and protein levels, as well as astrocytic glutamate uptake. Yet the detailed mechanism associated with the Mn-induced inhibitory effect on EAAT2 expression at the transcriptional level remains to be elucidated. Notably, Mn also potentiates the production of TNF-α (15), which is known to decrease the expression and function of EAAT2 (10).

Yin Yang 1 (YY1) is a ubiquitous transcription factor that plays an important role in the CNS during embryogenesis, differentiation, replication, and proliferation (16). YY1 can initiate, activate, or repress gene transcription, depending upon its interaction with available cofactors (17). For example, YY1 activation by TNF-α in

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myoblasts leads to inhibition of skeletal myogenesis (18). The functional role of YY1 in the brain is poorly understood. In rat neurons and astrocytes, YY1 binds to its putative recognition sequence within the β-site amyloid precursor protein (APP)-cleaving enzyme 1 (BACE1) promoter, leading to increased promoter activity (19). With respect to glutamate transporters, YY1 plays a role in EAAT1 (GLAST) repression as glutamate treatment increases YY1 DNA binding, decreasing glutamate uptake in chick Bergmann glia cells (20). YY1 has also been reported to regulate EAAT2 gene expression as astrocyte elevated gene 1 (AEG-1) is able to recruit YY1 to form a DNA binding complex to repress EAAT2 (21).

The objective of the present study was to identify the inhibitory mechanism of EAAT2 expression at the transcriptional level in facilitating the development of therapeutics for neurological diseases associated with impairment of glutamate transporters. For the first time, we demonstrate that YY1 represses EAAT2 promoter activity with recruitment of histone deacetylases (HDACs) as corepressors in primary astrocytes. Our findings also reveal that Mn not only increases YY1 expression via NF-κB but also enhances YY1 binding to the EAAT2 promoter, leading to the repression of EAAT2 promoter activity.

MATERIALS AND METHODS

Materials. Cell culture media (minimal essential medium [MEM], Dulbecco's modified Eagle's medium [DMEM], and Opti-MEM) and transfection reagents (Lipofectamine 2000) were purchased from Invitrogen (Carlsbad, CA). $MnCl_2$, valproic acid (VPA), and sodium butyrate (NaB) were obtained from Sigma-Aldrich (St. Louis, MO). Trichostatin A (TSA) was from Sigma-Aldrich (St. Louis, MO). Romidepsin (FK228) and suberoylanilide hydroxamic acid (SAHA; vorinostat) were from Selleck Chemicals. YY1 (sc-281), NF-κB (p65, sc-372), HDAC1 (sc-6298), β-actin (sc-1616), mouse IgG (sc-2025), rabbit IgG (sc-2027), and histone H3 (sc-10809) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Protein A/G-Sepharose beads and control and YY1 small interfering RNAs (siRNAs) were also from Santa Cruz. An RNA isolation kit was purchased from Qiagen (Valencia, CA). A luciferase reporter assay kit was obtained from Promega (Madison, WI).

Primary cultures of astrocytes. Animals were handled according to the approved protocol from the Meharry Medical College animal care and use committee following the established guidelines set by NIH for care and use of laboratory animals. Astrocytes were isolated from 1-day-old Sprague-Dawley rats as described previously (22). Briefly, after the meninges were removed, cerebral cortices were digested with dispase (Invitrogen, OR), and astrocytes were plated at a density of 1×10^5 cells/ml. The medium was changed after 24 h of initial plating, and the cultures were maintained at 37°C in a 95% air–5% CO_2 incubator for 3 weeks in minimum essential medium (MEM) supplemented with 10% horse serum, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. These cultures showed >95% positive staining for the astrocyte-specific marker, glial fibrillary acidic protein (GFAP). All experiments were performed 3 weeks postisolation.

Cell lysate preparation and Western blotting. Cells were treated with the designated compounds for the indicated time periods (see the figures and legends), followed by two washes with cold phosphate-buffered solution (PBS). Then, the cells were lysed with radioimmunoprecipitation assay buffer (RIPA) containing a protease inhibitor cocktail. The protein concentration in the lysate was determined with a bicinchoninic acid (BCA) assay, and 30 μg of protein samples was mixed with Laemmli sample buffer containing 5% β-mercaptoethanol and heated at 95°C for 5 min, followed by 10% SDS-PAGE. Western blot analysis was performed using primary antibodies for YY1, p65, HDAC1, histone H3 (1:1,000; Santa Cruz Biotechnology), and β-actin (1:5,000; Sigma-Aldrich), followed by secondary antibodies (1:3,000; anti-rabbit W4018– or anti-mouse IgG W4028–peroxidase conjugate [Promega] and anti-goat antibody [sc-2020; Santa Cruz]). The blots were detected with an enhanced chemiluminescence Western blotting detection kit (Pierce).

Immunocytochemistry procedures. To confirm YY1 expression in astrocytes, cells were double labeled with GFAP and YY1. The mouse polyclonal anti-GFAP (1:200) (sc-166458; Santa Cruz Biotechnology) and rabbit anti-YY1 (1:200) (sc-281; Santa Cruz Biotechnology) were used for the primary antibodies, and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit (1:100) (sc-3841; Santa Cruz) or fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse (1:100) (sc-2099; Santa Cruz) antibodies were used for secondary antibodies. The cell preparations were covered with Vector Shield mounting medium (Vector Laboratories) and observed under a Nikon confocal microscope (A1R laser scanning confocal).

Preparation of cytoplasmic and nuclear extracts. Cells were rinsed twice with ice-cold PBS and lysed in hypotonic buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$) containing 0.5% NP-40. Whole-cell contents were carefully collected with a cell scraper and transferred to a microtube. After 15 min on ice, the contents were centrifuged for 5 min at 2,500 rpm at 4°C. The supernatant cytoplasmic extract was collected, and the pellet (nuclei) was washed with hypotonic buffer without detergent. Then, the nuclei were incubated in hypertonic buffer (20 mM HEPES-KOH, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 25% glycerol) on ice for 30 min with periodic vortexing. After samples were spun at 20,000 × *g* for 10 min at 4°C, the supernatant nucleic extracts were collected and stored at −80°C until used.

co-IP. For coimmunoprecipitation (co-IP), the nuclear extracts were prepared as described above, and 2 μg of antibody was mixed with 400 μg of nuclear protein. After samples were rocked at 4°C for 1 h, 20 μl of protein A/G-agarose beads (Santa Cruz) was added and incubated overnight. The beads were washed three times with RIPA buffer, and 40 μl of 2× SDS sample buffer was added to elute the bound proteins from the beads; samples were then boiled at 95°C for 3 min. After a spinning step, the supernatant was collected and loaded on an SDS-PAGE gel for Western blotting. To rule out a possible DNA-protein interaction during the co-IP, nuclear extracts were treated with DNase I (Sigma-Aldrich).

Luciferase assay. Cells were grown in 24-well plates for 2 to 3 days before being transfected overnight with luciferase vectors. The pGL3 EAAT2 +282 (contains human EAAT2 promoter sequences from bp +282 to −954) plasmid vector was a generous gift from Albert Baldwin (University of North Carolina at Chapel Hill), and mouse YY1 wild-type and NF-κB mutant luciferase plasmids were from Denis Guttridge (Ohio State University) and Yang Shi (Harvard University). A luciferase reporter vector for NF-κB was from Clontech. The transfection of luciferase reporter vectors (0.5 μg) was performed with Lipofectamine 2000, and cells were switched to the growth medium containing 5% fetal bovine serum (FBS). After transfection, the cells were treated with the designated compounds in Opti-MEM for indicated time periods (see the figures and legends). Luciferase activity was measured with a Bright-Glo luciferase kit (Promega) according to the manufacturer's instructions and normalized to the protein content (determined by the Bradford method) (Bio-Rad). In all experiments, normalization was verified by cotransfection with firefly reporter pGL4.75 plasmids (Promega) carrying the *Renilla* luciferase reporter gene. The overexpression of various proteins with the plasmid vectors was achieved by overnight transfections with Lipofectamine 2000 in Opti-MEM. Equal amounts (100 ng) of empty vector pRC-RSV (for p65) and pcDNA (for YY1 and HDACs) were used as controls.

siRNA transfections. The control and YY1 siRNAs were obtained from Santa Cruz Biotechnology, and the transfections were performed with Lipofectamine 2000. Equal amounts of control and YY1 siRNAs (50 nM) were used. After 48 h posttransfection, the cells were lysed to carry out the luciferase assay and Western blotting.

Site-directed mutagenesis. The YY1 consensus binding sequence in the EAAT2 promoter was mutated by a QuickChange site-directed mutagenesis kit (Stratagene). The EAAT2 promoter (bp −954 to +282) subcloned into the pGL3 vector was used as the original template for mutation. The primer set used was 5′-CTC CCC GCC AAG CGC TAA CCC CGC GGG CGG-3′ and 5′-GAG GGG CGG TTC GCG ATT GGG GCG CCC GCC-3′. The mutant clones were confirmed by sequencing.

Quantitative real-time PCR. After treatment, cells were washed twice with ice-cold PBS, and total RNA was extracted using TRIzol reagent. A high-capacity cDNA reverse transcription kit (Applied Biosystems, USA) was used to transcribe 2 μg of RNA with poly(dT) oligonucleotides. Then, PCR targeting YY1 was performed with Supermix (Invitrogen) in a final volume of 30 μl with the following primer pairs: 5′-CTC CTG CAG CCC TGG GCG CAT C-3′ (YY1 forward) and 5′-GGT AAG CCC TTT AGC GCC TC-3′ (YY1 reverse); 5′-TCC CTC AAG ATT GTC AGC AA-3′ (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] forward) and 5′-AGA TCC ACA ACG GAT ACA TT-3′ (GAPDH reverse). After 30 cycles of amplification (denaturing at 94°C for 30 s, annealing at 55 to 64°C for 30 s, and extension at 72°C for 1 min) (iCycler; Bio-Rad), the samples were electrophoresed on a 1.5% agarose gel and visualized by ethidium bromide staining. GAPDH was used as an internal standard. For quantitative real-time PCR, a CFX96 real-time PCR detection system (Bio-Rad) was used to amplify YY1. The reactions were carried out in a total volume of 25 μl with mixtures containing 1 μg of cDNA template of each sample, 0.4 μM the appropriate primers, and RT2 SYBR green quantitative PCR (qPCR) Master Mix (SABiosciences/Qiagen). The PCR protocol consisted of one cycle at 95°C for 10 min and 40 cycles at 95°C for 15 s and at 60°C for 1 min. All samples were normalized relative to GAPDH. A Web-based PCR array data analysis (SA Biosciences/Qiagen) was used to analyze the data.

ChIP assays. Chromatin immunoprecipitation (ChIP) analysis was performed with an EZ-ChIP chromatin immunoprecipitation kit (Millipore) according to the manufacturer's instructions. Briefly, protein-DNA complexes were cross-linked with formaldehyde for 10 min at room temperature and sonicated to lengths of 100 to 500 bp. Then, 100 μl of supernatant was mixed with 900 μl of ChIP dilution buffer. After a preclearing step, 1% of the reaction mixture was saved for PCR amplification as input. The remainder was incubated overnight at 4°C with YY1 antibody (Santa Cruz) or control rabbit IgG (Millipore). After isolation and washing of antibody-containing complexes, DNA was extracted. PCR was done with the following primers for YY1: forward, 5′-GCG ACG ACG ACT ACA TTG-3′; reverse, 5′-TTC TTG CCG CTC TTC TTG CC-3′. PCR Products were resolved on 1% agarose gel and visualized under UV light.

EMSA. An electrophoretic mobility shift assay (EMSA) was performed using a LightShift Chemiluminescent kit from Thermo Scientific (Rockford, IL) according to the manufacturer's instructions. Briefly, 5 μg of nuclear extract from control or Mn-treated cells was incubated with biotin-labeled oligonucleotides containing YY1 consensus binding sites of the EAAT2 promoter for 20 min on ice. The DNA-protein complexes were resolved on 8% nondenaturing DNA polyacrylamide gels and transferred to nylon membranes. The complexes were detected using a Chemiluminescent Nucleic Acid Detection Module from Thermo Scientific. The primers pairs used for YY1 (EAAT2 promoter including the +34 YY1 consensus site) were 5′-CTC CCC GCC AAG CGC CAT CCC CGC GGG CGG-3′ and 5′-CCG CCC GCG GGG ATG GCG CTT GGC GGG GAG-3′. The oligonucleotides were purified by high-performance liquid chromatography (HPLC) and labeled with biotin (Operon Technologies).

DAPA. A DNA affinity purification assay (DAPA) was done using a Factor Finder Kit from Miltenyi Biotec, Inc. (Auburn, CA). Briefly, 1.5 μg of biotinylated oligonucleotides was incubated with 50 μg of nuclear extract in binding buffer for 20 min. The incubation was continued for another 10 min after the addition of 100 μl of Streptavidin microbeads. The reaction mixture was applied onto the microcolumn that was already equilibrated with two 100-μl washes of binding buffer. After four washes of 100 μl each with low-salt and high-salt buffers, proteins were eluted using 30 μl of elution buffer and analyzed by Western blotting.

ELISA. A rat TNF-α enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) was used to measure the TNF-α production in astrocytes according to the manufacturer's instructions. Briefly, cells grown in 24-well plates were treated, and the medium (100 μl/well) was collected, followed by the ELISA. The optical density was measured in a microplate reader set to 450 nm with wavelength correction at 570 nm.

Statistical analysis. The data are presented as the means ± standard errors of the means (SEM), and statistical analysis was carried out by one-way analysis of variance (ANOVA), followed by a Tukey *post hoc* test. To compare control and treated groups, statistical significance was set at a P value of <0.05. Each experiment was carried out in three or more independently isolated primary astrocyte preparations. The statistical unit is represented by the number of cultures used (not wells). Data analysis was carried out with GraphPad software (Graph Pad, San Diego, CA).

RESULTS

YY1 is a negative regulator of EAAT2, and it mediates Mn-induced repression of EAAT2. Several transcription factors are involved in the regulation of EAAT2 gene transcription. Although NF-κB is considered the main positive factor, it is also involved in negative regulation of EAAT2 expression (10). Its repressive action is regulated by N-myc recruitment (6). However, the negative regulatory mechanism for EAAT2 expression requires further study since mutation of N-myc consensus sites (−163 and −522) of EAAT2 decreased and overexpression of N-myc increased EAAT2 promoter activity (our unpublished data), indicating that N-myc also plays a positive role in EAAT2 regulation. These observations led us to posit that additional negative regulatory factors that repress EAAT2 promoter activity must exist.

YY1 mediates the AEG-1-induced repression of EAAT2 (21). Therefore, the role and mechanism of YY1 in negatively regulating EAAT2 transcription were ascertained. As shown in Fig. 1A, YY1 overexpression significantly decreased EAAT2 promoter activity ($P < 0.01$). Sequence analysis of the EAAT2 promoter showed a YY1 consensus site (+34), and mutation of this site increased EAAT2 promoter activity (Fig. 1B). To further confirm the repressive role of YY1 on EAAT2 promoter activity, YY1 siRNA was applied to knock down its endogenous expression. Transfection (48 h) with YY1 siRNA greatly reduced astrocytic YY1 mRNA and protein levels (Fig. 1C_2 and 1C_3), and EAAT2 promoter activity was significantly increased in the YY1 siRNA-transfected cells (Fig. 1C_1). Our earlier studies established that Mn reduced glutamate uptake by decreasing GLAST and GLT-1 mRNA and protein levels (13, 23, 24). Herein, we corroborated the Mn-induced decrease in astrocytic EAAT2 promoter activity in a time-dependent manner (Fig. 1D). Next, we determined if inactivation of YY1 reversed Mn repression of EAAT2 by using the YY1-site-mutated construct of the EAAT2 promoter. The results showed that the inhibitory effect of Mn on EAAT2 promoter activity was reversed in the YY1 mutant construct of EAAT2 (Fig. 1E). These results are consistent with YY1 negatively regulating EAAT2 and mediating the repressive effect of Mn on EAAT2 promoter activity.

Mn increases YY1 promoter activity and mRNA and protein levels. Given the direct involvement of YY1 in Mn-induced repression of EAAT2 promoter activity (Fig. 1E), we next examined if Mn regulates YY1 gene expression. This was driven by earlier observations that TNF-α activated the YY1 pathway in myoblasts (18) and that TNF-α, as well as Mn, repressed EAAT2 expression

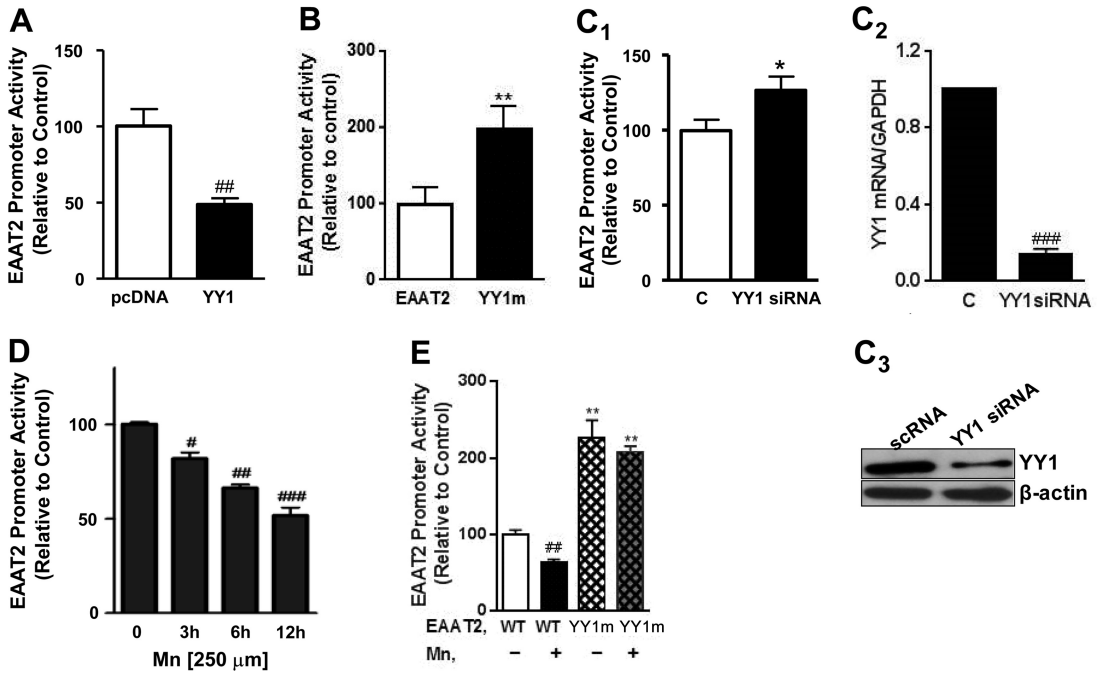

FIG 1 YY1 is a negative regulator of EAAT2, and it mediates Mn-induced repression of EAAT2. (A) Astrocytes were cotransfected overnight with 0.5 μg of EAAT2 luciferase plasmid and 0.1 μg of either the pcDNA control vector or YY1, followed by a luciferase assay to determine EAAT2 promoter activity as described in Materials and Methods. (B) The YY1 consensus site (+34) in the EAAT2 promoter was mutated by site-directed mutagenesis, and the promoter activity of the YY1 mutant (YY1m) of EAAT2 was compared with that of the wild-type EAAT2 by luciferase assay. (C) Astrocytes were transfected with YY1 siRNA or a scrambled control siRNA (scRNA) for 48 h, followed by a luciferase assay (C_1). The YY1 mRNA levels from qPCR (C_2) and YY1 protein levels from Western blotting (C_3) were measured to determine the efficiency of YY1 siRNA knockdown. (D) Astrocytes were treated with Mn (250 μM) for the indicated time periods, and EAAT2 promoter activity was measured by luciferase assay. (E) After overnight transfection with the wild-type or YY1 mutant EAAT2 promoter vector, astrocytes were treated with Mn (250 μM) for 6 h, followed by a luciferase assay. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$; *, $P < 0.05$; **, $P < 0.01$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$). WT, wild type.

(25). YY1 protein was expressed ubiquitously in astrocytes (Fig. 2A). Luciferase assays showed that Mn significantly increased YY1 promoter activity in a concentration-dependent manner (Fig. 2B). YY1 mRNA levels were also significantly increased in a time- and concentration-dependent manner following Mn treatment, with the maximal increase of YY1 mRNA expression at 3 h and 250 μM (Fig. 2C and D). Mn significantly increased the YY1 protein levels in whole-cell lysates as well as nuclear fractions of astrocytes at 6 h and 250 μM, possibly because time was required for transcription-translation (Fig. 2E), indicating that Mn increased YY1 expression at both the transcriptional and translational levels. The subcellular localization of YY1 has been shown to vary during various stages of the cell cycle (26). Further, YY1 is known to be abundantly expressed in cultured astrocytes (19). We observed comparable levels of YY1 expression in both the cytoplasm and nucleus, as evidenced by confocal image and subcellular fractionation (Fig. 2A and E).

Mn produces TNF-α, which activates YY1 in astrocytes. Although Mn may directly interact with the EAAT2 promoter by binding to proteins or enzymes which regulate EAAT2 promoter activity, it may also indirectly repress EAAT2 by increasing the release of levels of mediators, such as TNF-α. It has been previously reported that Mn potentiated lipopolysaccharide (LPS)-induced release of proinflammatory cytokines, such as TNF-α and interleukin-6 (IL-6) (10, 15). Accordingly, the ability of Mn to increase the astrocytic TNF-α level was determined by means of ELISA. As shown in Fig. 3A, Mn induced a significant increase in the astrocytic TNF-α level (Fig. 3A), and TNF-α reduced EAAT2 promoter activity (Fig. 3B). Notably, TNF-α (15 ng/ml) also increased YY1 promoter activity, as well as YY1 mRNA and protein levels (Fig. 3C, D, and E). These results indicate that Mn's effect on EAAT2 repression is mediated, at least in part, by TNF-α.

Mn induces YY1 binding to the EAAT2 promoter region. Since Mn increased YY1 protein levels (Fig. 2E), next we employed EMSA, DAPA, and ChIP assays to determine if Mn induced YY1 binding to the EAAT2 promoter region. As shown in Fig. 4A, EMSA revealed that Mn induced a significant increase of YY1 binding to the EAAT2 promoter compared with the control. This interaction was specific for YY1 as an excess of unlabeled YY1 oligonucleotides completely blocked the formation of this DNA-protein complex (Fig. 4A). Similarly, DAPA showed that Mn induced the binding of YY1 to its consensus site on the EAAT2 promoter (Fig. 4B). To confirm YY1 protein binding to the EAAT2 promoter (*in vivo* condition), a ChIP assay was performed. As shown in Fig. 4C, Mn induced YY1 binding to the astrocytic EAAT2 promoter region in a time-dependent manner using IgG as a negative antibody binding control (Fig. 4C). We also tested YY1 binding to the region of the EAAT2 promoter to which YY1 does not bind, but no YY1 binding to DNA was observed (data not shown). We also quantified Mn (250 μM)-induced binding of the EAAT2 promoter DNA to YY1 protein (Fig. 4D).

Mn and TNF-α regulate YY1 via NF-κB. The YY1 promoter contains two NF-κB binding sites (−170 and −155), but the

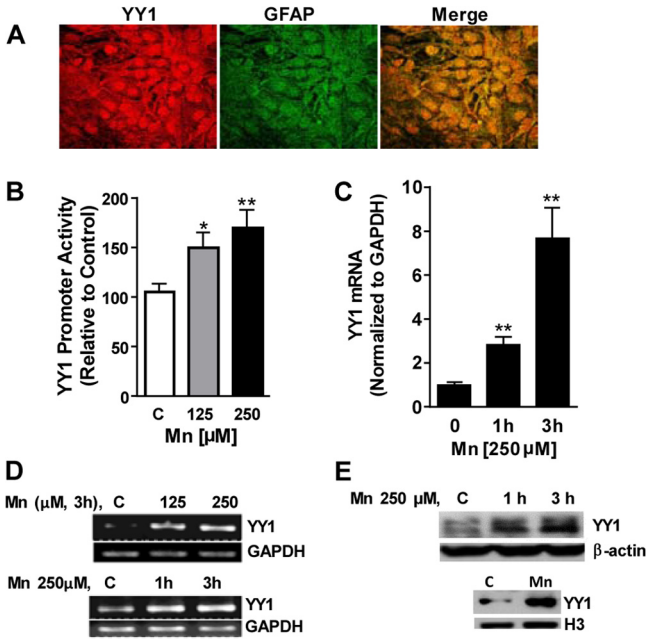

FIG 2 Mn increases YY1 expression. (A) Confocal image showing YY1 and GFAP expression in rat primary astrocytes. (B) After overnight transfection with the EAAT2 promoter vector, astrocytes were treated with Mn (0, 125, and 250 μM) for 6 h, followed by a luciferase assay. (C and D) Astrocytes were treated with Mn (with 250 μM for up to 3 h or for 3 h with up to 250 μM), followed by measurement of YY1 mRNA levels by qPCR (C) and conventional reverse transcription-PCR (D) using GAPDH as a control. (E) After treatment with Mn (250 μM), astrocytes were lysed, and YY1 protein levels were measured in whole-astrocyte lysates (top panel) or nuclear extract (bottom panel) by Western blotting. Equal amounts (30 μg) of cell lysates or nuclear extracts were loaded using β-actin and histone H3 as internal controls. *, $P < 0.05$; **, $P < 0.01$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

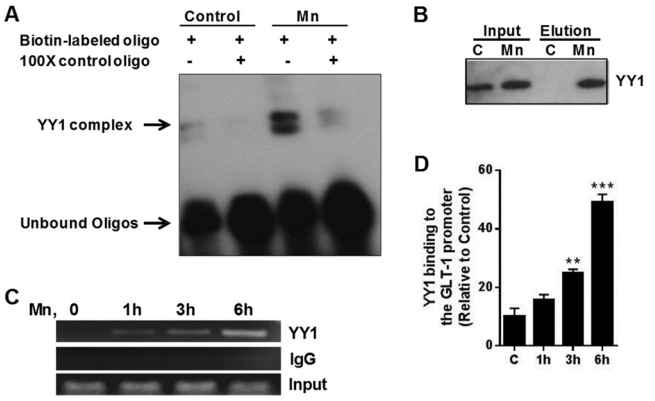

FIG 4 Mn recruits YY1 to the EAAT2 promoter. (A) EMSA was performed in nuclear extracts prepared from astrocytes treated with Mn (250 μM for 6 h) as described in Materials and Methods. The arrowhead shows the DNA-protein complex. (B) DAPA was performed with nuclear extracts prepared from astrocytes treated with Mn (250 μM for 6 h), and the YY1 consensus sequence-bound protein was subjected to Western blotting to probe YY1. As an input control (C), 10 μg of nuclear extract was used. (C) Astrocytes were treated with Mn (250 μM) for the indicated time periods, followed by a ChIP assay to determine YY1 binding to its consensus site in the EAAT2 promoter *in vivo*. (D) The PCR products were also quantified. **, $P < 0.01$; ***, $P < 0.001$ (Student's *t* test; $n = 2$).

−170 NF-κB consensus site is a functional site that plays a critical role in regulating YY1 promoter activity (18). To determine if NF-κB regulates astrocytic YY1 promoter activity, we tested the effect of NF-κB mutation on YY1 promoter activity. Mutation of the NF-κB binding site on the YY1 promoter (−170), in which the sequence was changed from GGGGGCCCCC to TTGGGCCCAA (mutated residues are underlined) reduced YY1 promoter activity significantly (Fig. 5A and D). In addition, the overexpression of the p65 subunit of NF-κB significantly increased wild-type YY1 promoter activity but did not increase the YY1 promoter activity in the NF-κB mutant (Fig. 5B and C). Most notably, Mn, as well as TNF-α, failed to increase YY1 promoter activity when the NF-κB sites of the YY1 promoter were mutated (Fig. 5E).

YY1 directly interacts with NF-κB p65 and overrides the NF-κB stimulatory effect. NF-κB activation represents the main mechanism for the positive regulation of EAAT2 expression, but negative EAAT2 regulators, such as TNF-α and Mn, also activate NF-κB (10, 25). This contradictory molecular event requires further study to provide a better understanding of the mechanism for the seemingly opposite directions of EAAT2 regulation in response to NF-κB activation (25). It has been previously reported that N-myc recruitment negatively regulates NF-κB on EAAT2 (10). However, under our experimental conditions, N-myc positively regulated EAAT2 promoter activity since mutation of its consensus sites in the EAAT2 promoter decreased EAAT2 promoter activity (unpublished data). This prompted us to explore other mechanisms that might mediate the inhibitory effect of NF-κB on EAAT2. We posited that YY1 might play a role in this process. As shown in Fig. 6A, overexpression of p65 alone increased EAAT2 promoter activity, but coexpression with YY1 decreased its activ-

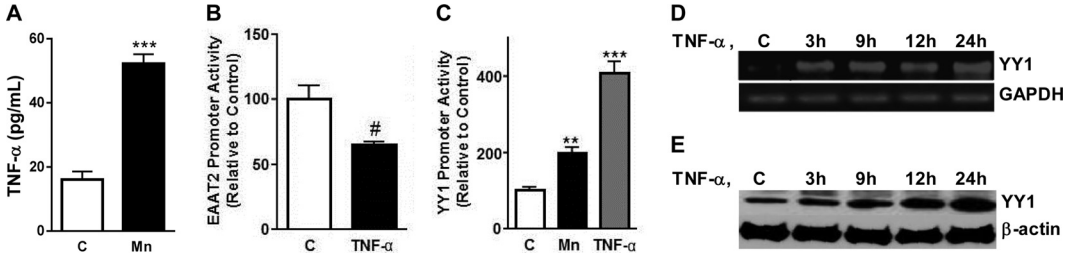

FIG 3 Mn treatment releases TNF-α in astrocytes. (A) Astrocytes were treated with Mn (250 μM) for 6 h, and the release of TNF-α was determined by ELISA. (B) After overnight transfection with EAAT2 promoter vector, astrocytes were treated with 15 ng/ml of TNF-α for 6 h, followed by a luciferase assay. (C) After overnight transfection with 0.5 μg of YY1 promoter, astrocytes were treated with Mn (250 μM) or TNF-α (15 ng/ml) for 6 h, followed by a luciferase assay. (D and E) Astrocytes were treated with TNF-α (15 ng/ml) for the indicated periods of time and YY1 mRNA (D) and protein (E) levels were measured using quantitative PCR and Western blotting, respectively. **, $P < 0.01$; ***, $P < 0.001$; #, $P < 0.05$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

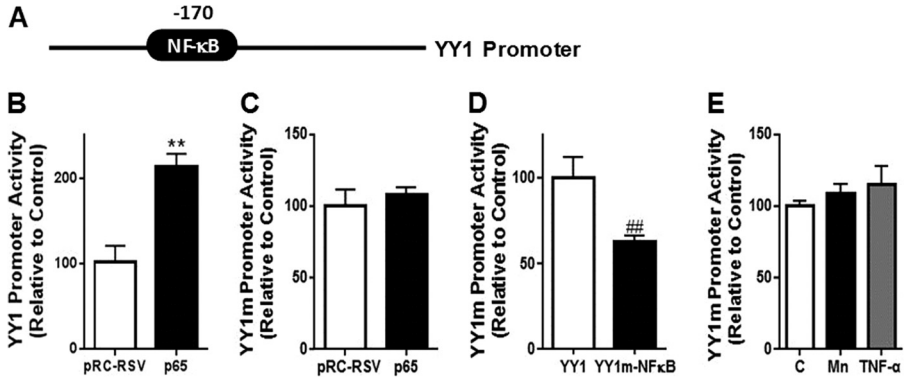

FIG 5 NF-κB regulates YY1 activation. (A) There is one critical NF-κB consensus site (−170) in the YY1 promoter. Astrocytes were transfected overnight with a wild-type (B) or an NF-κB mutant (C) YY1 luciferase plasmid (0.5 μg) and 0.1 μg of the control vector pRC-RSV or p65, and promoter activity was determined by luciferase assay. (D) Astrocytes were transfected with 0.5 μg of either the wild-type or NF-κB mutant (−170) YY1 promoter, followed by a luciferase assay. (E) After overnight transfection with an NF-κB mutant of the YY1 promoter vector, astrocytes were treated with Mn (250 μM) and TNF-α (15 ng/ml) for 6 h, followed by a luciferase assay. ##, $P < 0.01$; **, $P < 0.01$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

ity, indicating that the inhibitory activity of YY1 overrides the stimulatory effect of NF-κB. This also provides a plausible explanation for the repressive effect of TNF-α and Mn on EAAT2 regulation, specifically when TNF-α or Mn simultaneously activates both the positive p65 and the negative YY1 regulators. The results also showed that p65 and YY1 physically interacted and that Mn-treatment enhanced this interaction, as confirmed by coimmunoprecipitation (co-IP) (Fig. 6B).

HDACs serve as epigenetic corepressors with YY1. YY1 recruits epigenetic corepressors to exert its repressive gene regulation (18). Given the role of HDAC inhibitors in upregulation of glutamate transporter gene expression (27), we determined if HDACs play a corepressor role in YY1-induced EAAT2 inhibition. First, we studied the effect of overexpression of HDAC subtype members belonging to classes I and II on EAAT2 promoter activity. Overexpression of all HDACs (HDAC1 and -3 for class I and HDAC6 and -7 for class II) decreased EAAT2 promoter activity, with the highest suppression by HDAC7 (Fig. 7A). Moreover, coexpression of YY1 and HDACs further decreased EAAT2 promoter activity, indicating that HDAC classes I and II serve as corepressors with YY1 in inhibiting EAAT2 promoter activity (Fig. 7A). The direct physical association of YY1 with HDACs was confirmed by co-IP, as shown for HDAC1 and YY1, and Mn significantly enhanced this interaction within 1 h of treatment (Fig. 7B).

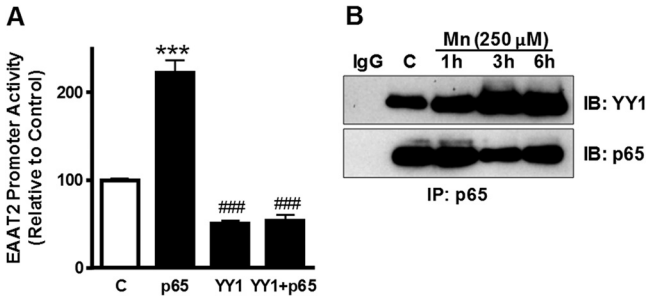

FIG 6 YY1 interacts with NF-κB p65, overriding the p65 effects. (A) Astrocytes were cotransfected overnight with the EAAT2 promoter vector and either YY1, p65, or both, followed by a luciferase assay. (B) Astrocytes were treated with Mn (250 μM) for the indicated time periods, followed by nuclear extract preparation and co-IP for YY1 and p65 as described in Materials and Methods. ###, $P < 0.001$; ***, $P < 0.001$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

HDACs suppress NF-κB p65 effects on EAAT2. As shown above, HDACs acted as corepressors of YY1 in EAAT2 regulation (Fig. 7), and, more importantly, YY1 directly interacted with NF-κB p65 to inhibit the p65 stimulatory effect on EAAT2 (Fig. 6). Accordingly, we examined if HDACs played a role in p65-induced EAAT2 regulation. We tested HDAC1 and HDAC4 as representatives of HDAC classes I and II, respectively. While NF-κB p65 overexpression alone enhanced astrocytic EAAT2 promoter activity (Fig. 8A), its stimulatory effect on EAAT2 promoter activity was completely abolished when p65 was coexpressed with HDAC1 or HDAC4 (Fig. 8B). HDAC1 directly interacted with p65 as shown by IP, and Mn also enhanced the interaction between p65 and HDAC1, albeit to a much lesser degree than that of YY1 with HDAC1 (Fig. 8C). To determine if YY1 mediates the HDAC1-p65 interaction, we performed the co-IP experiments after knocking down YY1 with siRNA. The results showed that YY1 facilitated the interaction of HDAC1 and p65 to some extent, but HDAC1-p65 interaction was mostly independent of YY1 since the knockdown of YY1 did not completely inhibit the interaction between the two proteins (Fig. 8D).

HDAC inhibitors reverse Mn-induced repression of EAAT2. Several studies have shown the neuroprotective effects of HDAC inhibitors in various neurodegenerative disease models (see reference 28 for a review). Moreover, HDAC inhibitors afford neuroprotection by upregulating glutamate transporters (27). Accordingly, we addressed the role of HDACs in EAAT2 regulation and Mn repression of EAAT2. Pharmacological HDAC inhibitors (HDACi) included trichostatin A (a classical HDAC inhibitor), sodium butyrate (pan-HDAC inhibitor), valproic acid, romidepsin (FK228), and SAHA (vorinostat). All HDACi significantly increased EAAT2 promoter activity (Fig. 9). In particular, SAHA and valproic acid exerted the highest stimulatory effects on EAAT2 promoter activity among all HDACi we tested. Notably, the Mn-induced decrease in astrocytic EAAT2 promoter activity was completely reversed by pretreatment with these HDACi (Fig. 9A and B).

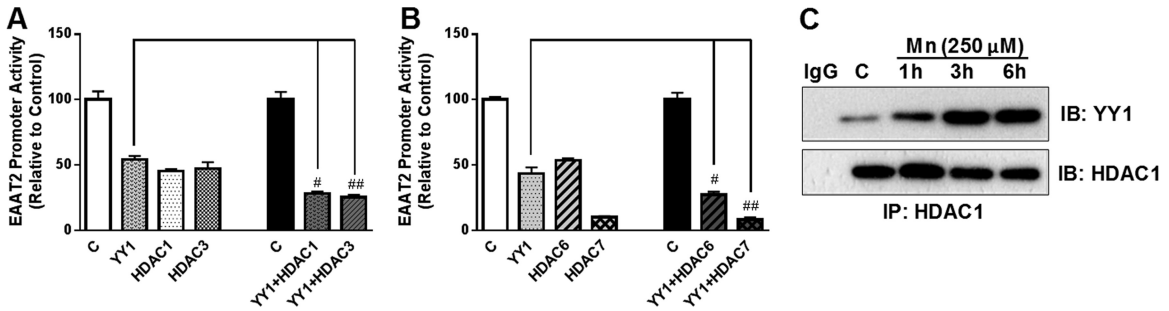

FIG 7 HDACs serve as corepressors of YY1. (A and B) Astrocytes were cotransfected overnight with the EAAT2 promoter vector and either YY1, HDACs, or both expression vectors, followed by a luciferase assay. (C) Astrocytes were treated with Mn (250 μM) for the indicated times, and nuclear extracts were prepared, followed by co-IP for YY1 and HDAC1. C, control; IB, immunoblotting. #, $P < 0.05$; ##, $P < 0.01$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

DISCUSSION

We investigated the role of YY1 in EAAT2 regulation. For the first time, we established that YY1 is a key negative transcription factor of EAAT2 and a mediator of TNF-α- and Mn-induced EAAT2 repression. Mn induced YY1 expression via NF-κB activation and enhanced YY1 binding to the EAAT2 promoter, leading to repression of EAAT2 promoter activity (Fig. 4). This effect is regulated, at least in part, via TNF-α, given that Mn led to the release of TNF-α, a negative regulator of EAAT2 (6). Moreover, YY1 directly interacted with NF-κB p65, thereby overriding the stimulatory effect of NF-κB on EAAT2 promoter activity (Fig. 6). This dominant effect of YY1 may explain the molecular mechanism by which Mn and TNF-α activate NF-κB, a positive regulator of EAAT2, yet still represses EAAT2 transcription. Epigenetically, HDACs served as corepressors of YY1 in EAAT2 promoter activity, augmenting YY1 repression of EAAT2 promoter activity. HDACs also directly interacted with NF-κB p65, reversing the positive regulation of NF-κB on EAAT2 promoter activity (Fig. 10).

EAAT2 functions as the predominant glutamate transporter in the CNS, maintaining extracellular glutamate at optimal levels and thus preventing excitotoxic neuronal injury (29). Given that reduction of EAAT2 expression and function is associated with various neurological disorders, the transcriptional mechanism of negative regulation of EAAT2 remains to be elucidated. Our findings indicate that the YY1 pathway might play a critical role in the negative regulatory mechanisms for EAAT2 expression and function. YY1 plays a role in glutamate-induced repression of GLAST (20). YY1 was recruited to the astrocyte elevated gene 1 (AEG-1) complex to repress EAAT2 promoter activity as well as expression in human primary astrocytes (28). Accordingly, we posited that YY1 is a critical transcription factor of EAAT2 repression, and both Mn and TNF-α increase YY1 promoter activity and mRNA and protein levels to repress EAAT2 transcription. Our findings provide strong evidence that YY1 is a key negative regulator of EAAT2 and explain the mechanism of TNF-α- and Mn-induced repression of EAAT2 when these activate both NF-κB and YY1 concomitantly. The increased interaction between NF-κB p65 and YY1 by Mn treatment further supports the idea that YY1 seizes NF-κB. YY1 interaction with NF-κB RelB in complex with the Oct-2 transcription factor on the IgH enhancer has been reported in B cells (30), indicating that YY1 might interact with the NF-κB subunits.

The regulation of YY1 promoter activity and expression has been shown to be dependent on NF-κB activation in prostate cancer cells and myoblasts (18, 31). Corroborating these reports, NF-κB plays an essential role in YY1 expression in astrocytes as mutation of the NF-κB binding consensus sites in the YY1 promoter abrogated YY1 promoter activity. TNF-α and Mn activated NF-κB, subsequently enhancing astrocytic YY1 expression. This activation occurred within hours (Fig. 3), indicating that activation of NF-κB at early time points via YY1 might lead to the neg-

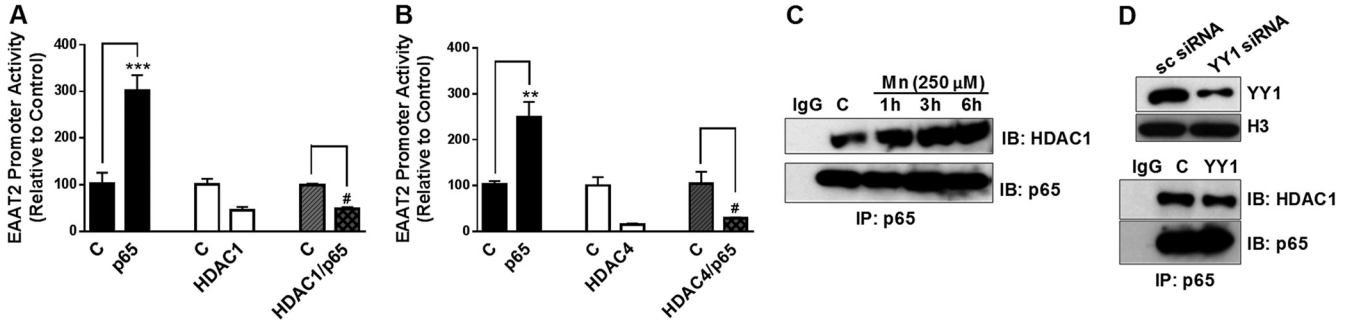

FIG 8 HDAC inhibits the stimulatory p65 effects on EAAT2 promoter activity. (A and B) Astrocytes were cotransfected overnight with either or both p65 and HDAC1 or HDAC4 expression vectors or their empty vectors (control) along with the EAAT2 promoter vector, followed by a luciferase assay. (C) The co-IP of HDAC1 and p65 was carried out using the nuclear extracts prepared from control and Mn-treated cells. (D) The co-IP of HDAC1 and p65 was performed using nuclear extracts which were prepared from astrocytes transfected with scrambled control (sc siRNA) and YY1 siRNAs for 48 h. Nuclear extracts were also blotted for YY1 to confirm the efficiency of knockdown with YY1 siRNA. Histone H3 was used as a loading control. #, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

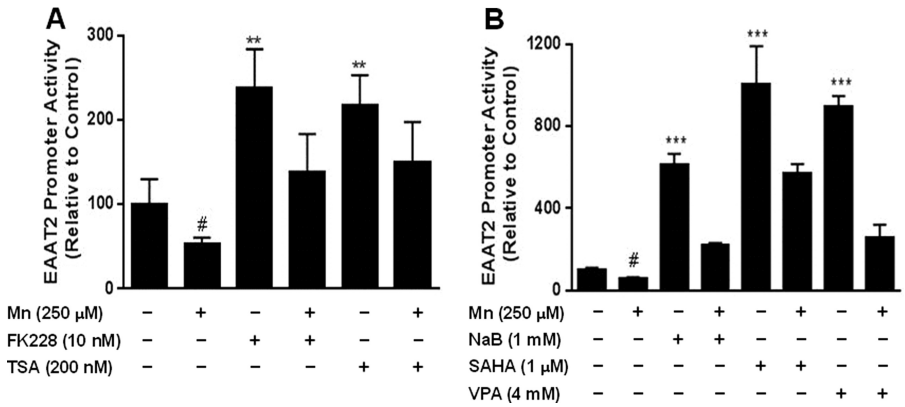

FIG 9 HDAC inhibitors increase and reverse Mn-induced repression of EAAT2 promoter activity in astrocytes. Astrocytes were exposed to vehicle (controls) or the following HDACi: romidepsin (FK228; 10 nM) and trichostatin A (TSA; 200 nM) (A) or sodium butyrate (NaB; 1 mM), SAHA (vorinostat; 1 μM), and valproic acid (VPA; 4 mM) (B) for 24 h. Mn, where indicated, was added at hour 18 during this incubation. #, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (ANOVA followed by Tukey's *post hoc* test; $n = 3$).

ative regulation of EAAT2 (Fig. 4). This also suggests that the molecular target of Mn/TNF-α-induced NF-κB activation is mainly linked to the YY1 pathway for the negative regulation of EAAT2 promoter activity. On the other hand, positive regulators, such as EGF, activate NF-κB to increase EAAT2 but require a considerably longer time, up to several days (6). This might be a critical molecular mechanism for NF-κB activation of EAAT2 promoter activity, explaining the role of NF-κB and YY1 in both positive and negative regulation of EAAT2 transcription.

YY1 regulates numerous genes by acting as a transcriptional initiator, activator, or repressor, functioning as a critical regulator in the development and progression of various cancers (32). However, the role of YY1 in the CNS remains poorly understood. It has been reported that glutamate altered formation of YY1 complexes with DNA, possibly modulating YY1-mediated gene regulation (33). YY1 has also been invoked in the progression of AD secondary to its binding to the BACE1 promoter, whose expression is increased in the AD brain (19). The role of YY1 in glutamate-induced inhibition of GLAST function in chick Bergmann glia (20) and in AEG-1-induced repression of EAAT2 expression in human glioblastoma cells (20, 21) further underscores the etiologic role of YY1 in triggering neurodegeneration. Our findings

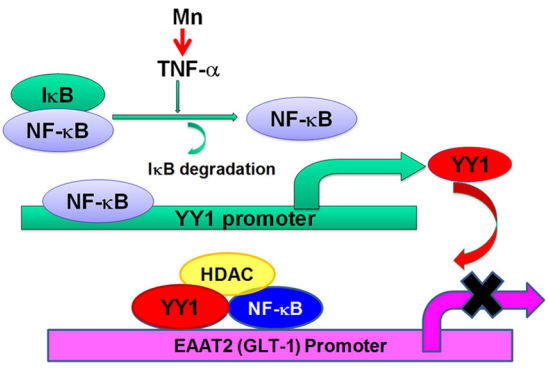

FIG 10 Proposed mechanism for Mn-induced repression of EAAT2. TNF-α is released by Mn, which activates the NF-κB pathway, followed by YY1 activation. The upregulation of YY1 represses EAAT2 using HDACs as corepressors. YY1 also physically interacts with NF-κB, inhibiting its positive regulation on EAAT2.

support the concept that YY1 is a key negative regulator of EAAT2 promoter activity and mediator of Mn- and TNF-α-induced repression of EAAT2. To our knowledge, this is the first report establishing the direct repressive role of YY1 in TNF-α repression on EAAT2 promoter activity.

Excitotoxic neuronal injury induced by elevated glutamate levels is considered one of the main mechanisms of Mn-induced neurotoxicity (34, 35). Our findings provide substantial evidence that YY1 is a mediator of Mn-induced repression of EAAT2. Mn-induced production of TNF-α, at least in part, appeared to mediate Mn's effect on this repression via YY1 activation. Mn has been shown to potentiate the LPS-induced release of TNF-α, which is a negative regulator of EAAT2 (6, 15). Accordingly, Mn-induced TNF-α might function to repress EAAT2 transcription via an autocrine mode upon release into the extracellular compartment. Moreover, TNF-α may contribute to neuronal injury by reduction of EAAT2 function via YY1 activation. Several neurodegenerative diseases are closely associated with both neuroinflammation and excitotoxicity (36). The astrocytic glutamate transporters, GLT-1 and GLAST, may represent a critical link between neuroinflammation and excitotoxic events. In particular, TNF-α might serve as the key factor to induce both neuroinflammation and excitotoxicity by inducing impairment of glutamate transporters in addition to contributing as a proinflammatory cytokine.

HDACs closely interact with astrocytic YY1 and NF-κB, leading to the modulation of EAAT2 promoter activity. HDACs 1 to 3 act as corepressors of YY1 (37). All tested class I and II HDACs (HDAC1, -3, -6, and -7) augmented YY1-induced EAAT2 repression, indicating that they serve as corepressors for YY1 in EAAT2 promoter activity. HDAC7 appears to be the most potent repressor, and, thus, it is our future plan to investigate the HDAC mechanisms associated with Mn-induced repression of EAAT2 via YY1. Mn enhanced the interaction between YY1 and HDAC1, suggesting that negative EAAT2 regulators such as Mn or TNF-α increase YY1 or/and HDAC1 expression, leading to enhanced interaction of YY1 with HDACs. It has been reported that TNF-α increased HDAC expression (38), indicating the possibility of Mn-induced HDAC expression via TNF-α. HDACs also directly interact with the positive EAAT2 regulator, NF-κB, and this interaction was

also enhanced by Mn, leading to repression of EAAT2 promoter activity and complete abrogation of the positive regulatory effect of p65 on EAAT2 promoter activity. Mn-induced interaction between HDAC and YY1 was stronger than that with NF-κB p65, suggesting that the YY1 pathway is the main mechanism for Mn-induced EAAT2 repression.

HDACs have been implicated in several forms of brain disorders, and HDAC inhibitors have emerged as promising therapeutics for various neurodegenerative diseases (28, 39). Therefore, exploring epigenetic regulatory mechanisms contributing to EAAT2 regulation is invaluable in identifying molecular targets of neuroprotectants to treat neurological disorders associated with impairment of glutamate transporters (40). HDACs are distributed ubiquitously, but HDACi alter only 2 to 10% of genes in transformed cells (41, 42). This selectivity may be a consequence of acetylation of a particular complex of histones and other proteins regulating gene expression (37). Various HDACi, which are used for either neurological disorders or cancers, in the present study increased astrocytic EAAT2 promoter activity and reversed the Mn-induced repression (Fig. 9). This is interesting because reduction of EAAT2 expression is observed in both excitotoxic neuronal injury and glioblastoma (24). A classical HDACi, trichostatin A, has been shown to enhance GLT-1 mRNA levels in C6 glioma cells (27). The mitigating effect of Mn-induced EAAT2 repression by HDACi is likely independent of counteracting Mn action because the reversing effect of EAAT2 promoter activity was significantly higher than the basal levels. Among inhibitors, SAHA appears to be the most effective HDACi in increasing EAAT2 expression and function. SAHA exerted neuroprotection against oxygen and glucose deprivation (OGD) insults and increased GLT-1 expression in isolated mouse optic nerve preparation (43). Valproic acid has been reported to increase GLAST expression in chick Bergmann glial cells (44). We found that valproic acid also increased EAAT2 promoter activity in astrocytes. These results suggest that HDACi have a high potential for treating various neurological disorders associated with the impairment of glutamate transporters, including neurodegenerative diseases and mental disorders such as depression in addition to Mn neurotoxicity.

Taking these results together, we propose a mechanistic model for the repressive effect of YY1 on EAAT2 promoter activity via NF-κB activation (Fig. 10). In this model Mn increases the release of TNF-α, which, in turn, enhances YY1 expression via activation of NF-κB. The epigenetic regulator HDACs are recruited as corepressors for YY1-induced EAAT2 repression. YY1 interacts with NF-κB, which also binds to HDAC. Notably, the inhibitory effect of YY1 on EAAT2 promoter activity is dominant over the stimulatory effect of NF-κB. Mn and TNF-α act in a similar mode in EAAT2 repression via activation of YY1. Combined, our novel findings establish that (i) YY1 is a crucial mediator in EAAT2 repression, (ii) YY1 expression is enhanced by Mn and TNF-α via NF-κB activation, and (iii) YY1 recruits HDACs as corepressors in EAAT2 repression. These findings shed insight on the negative regulatory mechanism of glutamate transporters and should facilitate the development of therapeutics for neurological disorders associated with impairment in glutamate transporter function.

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