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ARTICLE

Repeated treatment with electroconvulsive seizures induces HDAC2 expression and down-regulation of NMDA receptor-related genes through histone deacetylation in the rat frontal cortex

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Abstract

The enzymatic activity of histone deacetylases (HDACs) leads to a histone deacetylation-mediated condensed chromatic structure, resulting in transcriptional repression, which has been implicated in the modifications of neural circuits and behaviors. Repeated treatment with electroconvulsive seizure (ECS) induces changes in histone acetylation, expression of various genes, and intrabrain cellular changes, including neurogenesis. In this study, we examined the effects of repeated ECS on the expression of class I HDACs and related changes in histone modifications and gene expression in the rat frontal cortex. Ten days of repeated ECS treatments (E10X) up-regulated HDAC2 expression at the mRNA and protein levels in the rat frontal cortex compared with sham-treated controls; this was evident in the nuclei of neuronal cells in the prefrontal, cingulate, orbital, and insular cortices. Among the known HDAC2 target genes, mRNA expression of *N*-methyl-D-aspartate (NMDA) receptor signaling-related genes, including early growth response-1 (*Egr1*), *c-Fos*, glutamate receptor, ionotropic, N-methyl D-aspartate 2A (*Nr2a*), *Nr2b*, neuritin1 (*Nrn1*), and calcium/calmodulin-dependent protein kinase II alpha (*Camk2α*), were decreased, and the histone acetylation of H3 and/or H4 proteins was also reduced by E10X. Chromatin immunoprecipitation analysis revealed that HDAC2 occupancy in the promoters of down-regulated genes was increased significantly. Moreover, administration of sodium butyrate, a HDAC inhibitor, during the course of E10X ameliorated the ECS-induced down-regulation of genes in the rat frontal cortex. These findings suggest that induction of HDAC2 by repeated ECS treatment could play an important role in the down-regulation of NMDA receptor signaling-related genes in the rat frontal cortex through histone modification.

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Key words: ECS, HDAC, histone acetylation, synaptic plasticity genes.

Introduction

A family of enzymes called histone deacetylases (HDACs) mediate the deacetylation of histone tails, which, through the enzymatic activity of HDACs, leads to a condensed chromatin structure that results in transcriptional repression (Grozinger and Schreiber, 2002; de Ruijter et al., 2003). Dynamic regulation of histone acetylation

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by the balance between histone acetyltransferases (HATs) and HDACs may affect the expression of a wide variety of genes (Graff and Tsai, 2013). Accumulating evidence suggests that HDACs play important roles in synaptic plasticity, learning, memory, and adult neurogenesis (Levenson and Sweatt, 2005; Tsankova et al., 2007; Abel and Zukin, 2008; Jiang et al., 2008; Sun et al., 2011). There are 18 HDAC enzymes in the mammalian genome, and they are generally divided into four classes: I, II, III, and IV (Yang and Seto, 2008). Although structural homology and common catalytic mechanisms may translate into functional redundancy of HDAC isoenzymes, individual HDACs have different physiological functions (Kramer, 2009). In particular, class I HDACs have been implicated in synaptic plasticity and neuroprotective actions in the brain (Fischer et al., 2007; Nott et al., 2008; Guan et al., 2009).

Recent studies have indicated that changes in histone acetylation and related HDACs are implicated in the pathophysiology of psychiatric disorders and the mechanisms of actions of psychotherapeutic agents (Tsankova et al., 2007; Boks et al., 2012). Valproic acid, a mood stabilizer used in the treatment of bipolar disorder, is a HDAC inhibitor (Gottlicher et al., 2001; Phiel et al., 2001). Altered histone acetylation and related regulatory mechanisms in the post-mortem brains or blood cells of patients with schizophrenia have been reported (Akbarian et al., 2005; Sharma et al., 2006, 2008; Gavin et al., 2009; Tang et al., 2011), and antipsychotics, including clozapine and haloperidol, affect histone acetylation in the brain (Li et al., 2004; Guidotti et al., 2009; Kurita et al., 2012). HDAC2 was induced in the brain by chronic treatment with clozapine (Kurita et al., 2012), and the antidepressant fluoxetine increased expression of HDAC2 in the rat brain (Cassel et al., 2006). HDAC5 has been reported to be involved in the stress-mediated induction of depressive behaviors and antidepressant actions (Tsankova et al., 2006; Renthal et al., 2007). Various antidepressants may induce brain-region-specific changes in expression levels of different HDAC isoforms (Ookubo et al., 2013). Additionally, HDAC inhibitors induce antidepressant-like behavioral changes (Schroeder et al., 2007; Covington et al., 2009).

Electroconvulsive seizure (ECS) is an animal model of electroconvulsive therapy (ECT), which has been used in the treatment of depression as well as bipolar disorder and schizophrenia (Mukherjee et al., 1994; Tharyan and Adams, 2005; Lisanby, 2007). Repeated treatments are required to induce the therapeutic actions of ECT, and repeated ECS treatments have been reported to induce cellular proliferation and neurogenesis, synaptic modifications, increased expression of neurotrophic factors, changes in the activity of intracellular signaling molecules, and anti-apoptotic activity (Merkl et al., 2009). Repeated ECS treatments induce changes in the expression of various genes in the brain (Newton et al., 2003; Altar et al., 2004; Ploski et al., 2006; Segi-Nishida, 2011), which result in the long-term plastic changes induced by ECS.

Chromatin modifications could play crucial roles in the altered expression of various genes as a result of ECS. Previously, we reported that repeated ECS treatment decreased the expression of early growth response 1 gene (*Egr1*) and *c-Fos*. The inducibility of these genes in response to cocaine was also attenuated, despite the activation of an upstream kinase, extracellular signal-regulated kinase 1/2 (ERK1/2), in the rat frontal cortex (Park et al., 2011). These findings suggest that epigenetic mechanisms blocking the inducibility of these genes could be involved in the attenuated response of immediate early genes (IEGs) to stimuli. In fact, repeated ECS treatments induced down regulation of *c-Fos*, and histone deacetylation in the promoter region of *c-Fos* has been reported (Tsankova et al., 2004). Several studies have also indicated that ECS affects histone modifications in the brain (Crowe et al., 2011; Dyrvig et al., 2012; Fryland et al., 2012). These findings suggest that histone modifications could play important roles in ECS-induced gene expression changes. However, the regulatory mechanism(s) responsible for these histone modifications as yet remain unknown (Fig 1).

In this study, we investigated the effects of repeated ECS treatments on the expression of class I HDACs in the rat frontal cortex, and found that both *Hdac2* mRNA and HDAC2 protein level increased. Among the known HDAC2 target genes (Guan et al., 2009), mRNA expression and promoter histone acetylation of *Egr1, c-Fos*, neuritin1 (*Nrn1*), glutamate receptor, ionotropic, N-methyl D-aspartate 2A (*Nr2a*), glutamate receptor, ionotropic, N-methyl D-aspartate 2B (*Nr2b*), and calcium/calmodulin-dependent protein kinase II alpha (*Camk2α*) decreased after repeated ECS treatments. HDAC2 occupancy on their promoters was also increased, and treatment with an HDAC inhibitor recovered the ECS-induced down-regulation of these genes. These findings indicate that HDAC2 may play an important role in the ECS-mediated changes in gene expression in the brain.

Method

Animals

Animals were treated in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Formal approval to conduct this experiment was obtained from the animal subjects review board of Seoul National University Hospital. Male Sprague–Dawley rats (150–200 g) were housed for 1 wk before the experiments and maintained under a strict 12/12 h light/dark cycle (lights on 08:00 hours) with food and water available *ad libitum*.

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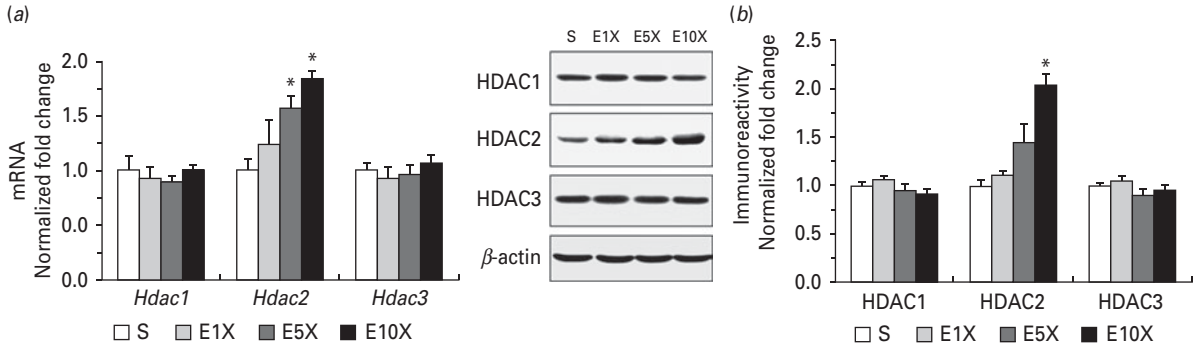

Fig. 1. Repeated ECS treatments induce HDAC2 mRNA and protein expression in the rat frontal cortex. (*a*) mRNA expression of class I *Hdacs* (*Hdac1, Hdac2*, and *Hdac3*) was analyzed with qRT-PCR, and values relative to the sham control (S) are presented. The *Hdac2* mRNA level was significantly increased in the rat frontal cortex 24 h after repeated ECS treatments for 5 d (E5X) and for 10 d (E10X). (*b*) Representative immunoblots and quantified immunoblot values of class I HDACs (HDAC1, HDAC2, and HDAC3) from the rat frontal cortex are presented. HDAC2 immunoreactivity was increased in the rat frontal cortex by E10X. The immunoreactivity value was quantified using a densitometric analysis of band intensity and is presented as relative optical density (OD) *vs.* the control. Values are presented as means±S.E. *, $p<0.05$ ($n=4$–8 for each treatment group).

ECS and sodium butyrate treatments

ECS was administered to the rats, and an ECS-induced seizure was validated as described previously (Kim et al., 2010). Sham-control animals were handled in the same fashion as the ECS treated group, but no electric current was delivered. Rats were divided into four groups for ECS treatments: sham treatment only for 10 d (S), sham treatment for 9 d then ECS on the 10th day (E1X), sham treatment for 5 d then ECS daily for 5 d (E5X), and ECS daily for 10 d (E10X). All treatments were administered once a day at the same time (12:00–13:00). The rats were decapitated 24 h after the last treatment. For the experiments on the effect of the HDAC inhibitor, 1200 mg/kg of sodium butyrate (NaBu; Sigma-Aldrich, USA) or normal saline vehicle were injected intraperitoneally (i.p.) into rats 30 min before ECS treatment during the last 4 d of E10X and 30 min before decapitation for a total of five times. Then, the frontal cortex was dissected and used for further analyses. The NaBu dose was determined based on previously published studies (Levenson et al., 2004; Fischer et al., 2007; Kim et al., 2011; Stafford et al., 2012). To inhibit ECS-induced HDAC2 activity, NaBu was injected after E5X, when a trend of increase in HDAC2 protein level was observed, for 5 d. The effects of NaBu were confirmed by analyzing histone acetylation and HDAC2 activity as described below.

Quantitative real-time PCR

Quantitative real-time PCR (qRT-PCR) was performed as described previously (Park et al., 2011). Briefly, total RNA was extracted from the frontal cortex using the TRI reagent (Molecular Research Center, USA). Then, 1 μg total RNA was reverse-transcribed to cDNA using the Superscript II RT system (Invitrogen, USA). Quantitative real-time PCR was performed using an ABI PRISM 7500 instrument (Applied Biosystems, USA) with the SYBR Green PCR Real-time PCR Master Mix (Toyobo, Japan). Relative amounts of all mRNAs were calculated by the comparative Ct method using the $2^{-\Delta Ct}$ equation. The ΔCt was the difference in the Ct values derived from the target gene and the *β-actin* control, whereas $\Delta\Delta Ct$ was calculated by the formula $\Delta Ct=\Delta Ct-\Delta Ct$ of the control group (sham or sham of CT8). The primer sequences for quantitative real-time PCR are summarized in Table 1.

Immunohistochemistry

Immunohistochemistry was performed using a free-floating method. Rats were anesthetized with urethane (1.5 g/kg, i.p.) 24 h after the last ECS treatment and perfused intracardially with 0.1 M PBS (pH 7.4) followed by 4% paraformaldehyde (Sigma-Aldrich) in 0.1 M PBS (pH 7.4). Brains were sectioned at 25 μm on a cryostat (Leitz, Germany), then immediately immersed in a cryoprotectant, 50% glycerol in 0.1 M PBS. Immunohistochemistry was performed with the ABC system (Invitrogen). Sections were washed with and incubated in 0.3% H_2O_2 for 30 min to quench endogenous peroxidase activity. After extensive washing with 0.1 M PBS, sections were blocked with 5% normal goat serum at room temperature for 30 min, then incubated overnight with primary antibodies against HDAC2 (Santa Cruz Biotechnology, USA) at a dilution of 1:500 at 4 °C. Sections were incubated with biotinylated secondary antibodies and then incubated with HRP-conjugated streptavidin. Signals were detected using DAB substrate. Subsequently, sections were mounted with DPX mountant (Fluka, Switzerland). Images of the prefrontal, orbital, cingulate, and insular cortices were obtained using an Olympus microscope equipped with a Leica DFC280 digital camera and software (Leica Application Suite V3, Germany), and all HDAC2-positive cells in 300×400 μm areas of each brain region were manually counted for subsequent quantitative analysis.

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Table 1. Primers for qRT-PCR

	Forward (5′–3′)	Reverse (5′–3′)
Hdac1	AGTATAATGTCCCCAGGGACCA	CTGAAAAGGGGCCCCAGAAG
Hdac2	CGGCAAGAAGAAAGTGTGCT	TCCATCGAACACTGGACAGT
Hdac3	GAGCCTTAATGCCTTCAACG	AGGTAGAAGGCTTCCTGAAC
Egr1	CTTCGCTCACTCCACTATCC	GATGAGTTGGGACTGGTAGG
c-Fos	GGAATTAACCTGGTGCTGGA	TGAACATGGACGCTGAAGAG
Cdk5r1	AGGCCACACTGTTTGAGGAT	AGGCAGTACCGAGATGATGG
Homer1	AGGCAGCTGAGTCCTTCAGA	AAACCGTCAAAGACGGACTG
Nrxn1	GGACGTTTCCTTACGGTTCA	TCCAGTCGAAGGGTATCTGC
Nrxn3	CATTGTGGAGCCAGTGAATG	GATTGTCACCTGCCGAAGAT
Cbp	GCTCCGTCTTTGCTCAACTC	AACACATGGCTTGGGACTTC
Glur1	AACCACCGAGGAAGGATACC	TGAGGCGTTCTGATTCACAG
Nrn1	CTCTTACGGATTGCCAGGAA	TCGAATAAGCTGCCTTGGAT
Nr2a	GTCTGGAGGACAGCAAGAGG	TCCCGATAACTAAGCGTTGG
Nr2b	TCCGTCTTTCTTATGTGGATATGC	CCTCTAGGCGGACAGATTAAGG
Camk2α	AGATGTGCGACCCTGGAATGAC	CGGGACCACAGGTTTTCAAAATAG
Npy	AGATACTACTCCGCTCTGCGA	GGCATTTTCTGTGCTTTCTCT
β-actin	CCTCTGAACCCTAAGGCCAA	AGCCTGGATGGCTACGTACA

Double-label immunofluorescence

Immunofluorescence was performed as described previously (Kim et al., 2013). The sections were blocked for 1 h with 3% BSA in TBST and then incubated with a specific antibody against HDAC2 (Santa Cruz Biotechnology), with an antibody against neuronal nuclear protein (NeuN; Chemicon, USA), or with an antibody against glial fibrillary acidic protein (GFAP; Chemicon) at 4 °C overnight. After washing in TBST, the sections were incubated with secondary antibodies (1:200 for Alexa Fluor 488 and 555, Molecular Probes, USA) for 1 h at room temperature. The sections were then incubated in 4′,6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) to stain nuclei. After mounting with mountant (Biomeda, USA), sections on slides were analyzed using a Meta confocal microscope (Model LSM 510; Carl Zeiss Micro Imaging Inc., Germany).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed according to the manufacturer's protocol with the EZ-magna ChIP kit (Millipore, USA) with some modifications. The frontal cortex was minced to ~1 mm-sized pieces in 0.1 M PBS containing protease inhibitors (Sigma-Aldrich), then immediately cross-linked for 15 min at room temperature in 1% paraformaldehyde. The cross-linking reaction was stopped by adding glycine to a final concentration of 0.125 M and incubating for 5 min at room temperature. The minced tissues were collected by centrifugation (800 *g*, 5 min, 4 °C) and washed three times with cold 0.1 M PBS containing protease inhibitors. Then, the tissue was homogenized in cell lysis buffer (10 mM Tris, 10 mM NaCl, and 0.2% NP-40) and incubated for 15 min at 4 °C with mild agitation. The homogenate was centrifuged (800 *g*, 5 min) to collect nuclei. The nuclear pellet was homogenized in nuclear lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1). Then, the extracted chromatin was sheared to 500–1000 bp using Bioruptor sonicator (Cosmobio, Japan). Each sample was sonicated five times on ice for 30 s periods separated by intervals of 60 s. The optical density of each sample was determined, and equal amounts of chromatin (10–15 μg) were diluted with ChIP dilution buffer containing 0.01% SDS, 1.1% Triton Z-100, 1.2 mM EDTA, 167 mM NaCl, and 16.7 mM Tris–HCl, pH 8.1 to a final volume of 500 μl. Then, 1% of the final volume was saved as input for the later normalization. The chromatin solution was immunoprecipitated overnight at 4 °C with 40 μl of protein A magnetic beads (Millipore) and 5 μg of specific antibodies against H3 acetylated at Lys9 and Lys14 (Millipore); H4 acetylated at Lys5, Lys8, Lys12, and Lys16 (Millipore); and HDAC2 (Abcam, USA). As a control, samples were immunoprecipitated with 5 μg of nonimmune rabbit IgG (Millipore). After immunoprecipitation, beads were sequentially washed for 5 min at 4 °C with low-salt buffer, high-salt buffer, LiCl buffer, and TE buffer. The immunoprecipitated DNA was then eluted by incubating in 100 μl of Elution buffer (0.1 M $NaHCO_3$ and 1% SDS) containing 10 μg proteinase K (Sigma-Aldrich) at 62 °C for 2 h with rotation. The eluted DNA was purified by using the columns and buffers contained in the kit (Millipore) or using PCR DNA fragment extraction kit (RBC Bioscience, Taiwan) and finally redissolved in 50 μl of PCR-grade water. The levels of specific histone modification and HDAC2 binding at each gene promoter region were determined by qRT-PCR as described above. Primer pairs for specific ~150 bp segments corresponding to each gene promoter region (regions within 500 bp

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Table 2. Primers for ChIP-qRT-PCR

Genes	Forward (5′–3′)	Reverse (5′–3′)
Egr1	ACCACCCAACATCAGCTCTC	GTGGGTGAGTGAGGAAAGGA
c-Fos	TTCTCTGTTCCGCTCATGACGT	CTTCTCAGTTGCTAGCTGCAATCG
Nrn1	AAAATCAATCCGTGTCATCC	GAGCTGGGAATGGTTCACTC
Nr2a	GGCTGGATGAGGTCTGAGAG	CGCAGCCTCTCTCCACATAG
Nr2b	ATGCTCTACGGAGGGACAGA	TCTTCAGCAATTACAGATTGGA
Camk2α	AGCTCGTCAATCAAGCTGGT	GGACTAGGACTGGGATGCTG
Npy	CAGTGTCTGGTCCCTACAGA	CTGAGAGGAGAAGGGAACAT
Gapdh	AGCAGCTTTCTGGTTTCTGG	ATGAGGGTTCCCAGGATAGG

upstream from the transcription starting site) were used, which were based on methods used in previous related reports (Tsankova et al., 2004; Freeman et al., 2008). The primers could reflect the proximal promoter region within 500 bp upstream from the transcription start site. Relative quantification of the template was performed as described previously (Chakrabarti et al., 2002). Average Ct values (^{average}Ct of IP) were obtained by averaging triplicate measurements for each sample, and the ^{net}Ct was calculated by substracting ^{average}Ct of the input from ^{average}Ct of IP. The ^{net}Ct of each gene was normalized ($^{norm\text{-}net}$Ct) with ^{net}Ct of Gapdh by substracting ^{net}Ct of IP from ^{net}Ct of GAPDH in histone modification experiments. The ^{net}Ct of each gene was normalized ($^{norm\text{-}net}$Ct) with ^{net}Ct of IgG by substracting ^{net}Ct of IP from ^{net}Ct of IgG in HDAC2 binding experiments. The relative amount of each sample was determined by raising 2 to the $^{norm\text{-}net}$Ct power. Primer sequences for ChIP-qRT-PCR are summarized in Table 2.

Histone extraction

Frontal cortex tissues were homogenized in ice-cold lysis buffer (pH 7.4) containing 10 mM Tris-HCl, 5 mM NaF, 1 mM Na_3VO_4, 1 mM EDTA, 1 mM EGTA, and 320 mM sucrose, and then the homogenates were centrifuged (800 *g*, 10 min, 4 °C). Pelleted nuclei were resuspended in 500 μl of 0.4 N H_2SO_4 and incubated on ice for 30 min and then centrifuged (14000 *g*, 10 min, 4 °C). The supernatant was transferred to a fresh tube, and proteins were precipitated with 250 μl of 100% trichloroacetic acid (final concentration of 33%) containing 4 mg/ml deoxycholic acid (Sigma-Aldrich) on ice for 30 min. The protein pellet was washed with ice-cold acidified acetone (0.1% HCl) followed by ice-cold acetone for 5 min each. Protein precipitates were collected between washes by centrifugation (14000 *g*, 5 min). The resulting purified proteins were resuspended in 10 mM Tris (pH 8.0). Protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad, USA). To assess histone post-translational modifications, 1 μg of histone extracts was separated by SDS-PAGE on a 12% resolving and 4% stacking gel. The rest of the procedures followed the immunoblot analysis procedure.

Immunoblot analysis

Immunoblot analyses were performed as described previously (Kim et al., 2008, 2013). Antibodies against HDAC1, HDAC2, HDAC3 (Abcam), and β-actin (Sigma-Aldrich) were used at dilutions of 1:1000 to 1:5000. For histone modification analysis, antibodies against ac-Lys9/14-H3, ac-Lys-H4 (Millipore), H3, and H4 (Cell signaling Technology, USA) were used. Blots were incubated with primary antibody overnight at 4 °C. Subsequently, the membranes were incubated with HRP-conjugated secondary antibody (Zymed Laboratories, USA), and the signals were detected using an enhanced chemiluminescence system (Pierce, USA). Immunoblot signals were quantified with the TINA program (v.2.10 g; Raytest, Germany). At least four independent experiments were performed.

HDAC2 activity assay

An HDAC2 activity assay was performed using an HDAC2 activity fluorometric assay kit (Biovision, USA) according to the manufacturer's instructions and previous reports (Marwick et al., 2004; Huang et al., 2012a). In brief, HDAC2 was immunoprecipitated from the frontal cortex extract by incubation with an HDAC2-specific antibody at 4 °C overnight. Each tissue extract was incubated with rabbit IgG as a negative control and background control. After incubation, the antibody–HDAC2 complex was captured by incubation with a protein–A/G slurry for 1 h at 4 °C. For the HDAC2 activity assay, the precipitated HDAC2 was incubated in HDAC assay buffer containing HDAC substrate at 37 °C for 1 h. Then the developer was added to the reaction mixtures and the mixtures were incubated at 37 °C for 30 min. To measure HDAC2 activity, AFC (7-amino-4-trifuluoromethyl coumarin) was used as a standard. After final incubation, the reaction mixtures and AFC standard were transferred to a 96-well black opaque plate and the fluorescence was read using Spectramax M5 (Molecular Devices, USA).

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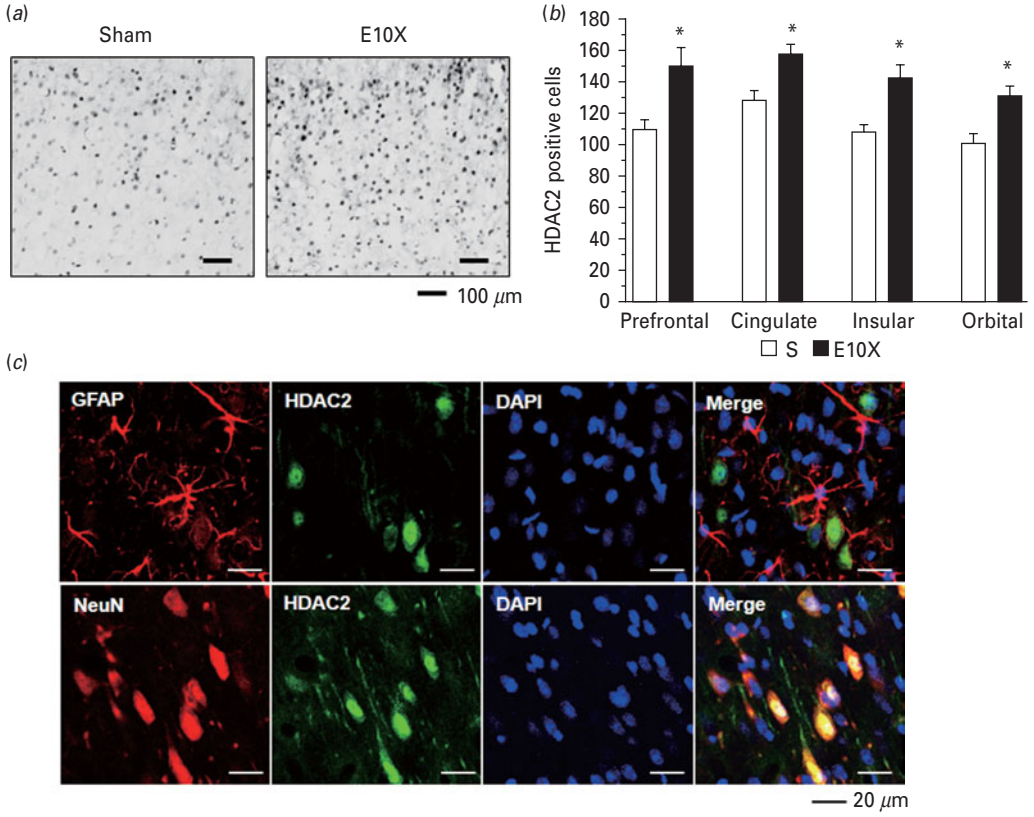

Fig. 2. Increased immunostaining for HDAC2 co-localized with immunofluorescence of Neu-N in the rat frontal cortex after repeated ECS treatments. (*a*) Representative microscopic images taken from sections stained for HDAC2 in the rat prefrontal cortex (Fr) (Paxinos and Watson, 1998). All captured images were obtained 24 h after the last of 10 daily ECS treatments (E10X). (*b*) The number of positive cells for HDAC2 was counted and was found to be increased in prefrontal (Fr), cingulate (Cg), insular (AI), and orbital (LO and VLO) cortical regions (Paxinos and Watson, 1998) after E10X. Values are presented as means±S.E. *, $p<0.05$ ($n=4$ for each treatment group). (*c*) Immunofluorescence of HDAC2 was co-localized with that of Neu-N (red) and DAPI (blue) in the rat prefrontal cortex (Fr) (Paxinos and Watson, 1998). Magnification bar=100 (*a*) and 20 μm (*c*).

Statistical analyses

Immunoblot results are expressed as relative optical density units (ODs), and results of qRT-PCR, ChIP-qRT-PCR, and the HDAC2 activity assay are expressed as fold changes relative to controls. All results are presented as means±S.E. The mean relative ODs of immunoreactivity and fold changes were compared with the control using an independent *t*-test or one-way analysis of variance (ANOVA), followed by a *post-hoc* LSD test. All tests were performed using the SPSS software (v.19.0 for Windows; SPSS, Inc., USA). p values <0.05 were deemed to indicate statistical significance.

Results

Repeated ECS treatments induce HDAC2 expression in the rat frontal cortex

Expression changes of class I HDACs after ECS treatments in the rat frontal cortex were investigated. Class I HDACs consist of HDAC1, 2, 3, and 8. Among them, HDAC8 has been reported to be barely expressed in the rat cerebral cortex (Broide et al., 2007). We also found that *Hdac8* mRNA was barely detectable in the rat frontal cortex (data not shown). The *Hdac2* mRNA level was significantly affected by ECS treatments ($F=9.434$, $p<0.001$): *Hdac2* mRNA level was significantly increased by E5X ($p=0.002$) and E10X ($p<0.001$). However, the mRNA levels of *Hdac1* and *Hdac3* were not changed by ECS treatments. The protein level of HDAC2 was also significantly affected by ECS treatments ($F=4.962$, $p=0.038$), in that E5X induced a trend toward an increase ($p=0.073$) and E10X induced a significant increase ($p=0.016$) in HDAC2 protein levels. HDAC1 and HDAC3 protein levels were not significantly affected by ECS treatments. Taken together, these results indicate that repeated ECS treatments induce an increase in HDAC2 levels in the rat frontal cortex.

Increased HDAC2 immunoreactivity after E10X was localized in the nucleus of neurons in the rat frontal cortex.

To investigate the detailed anatomical localization of HDAC2-positive cells within the frontal cortical regions after E10X, an immunohistochemical analysis was

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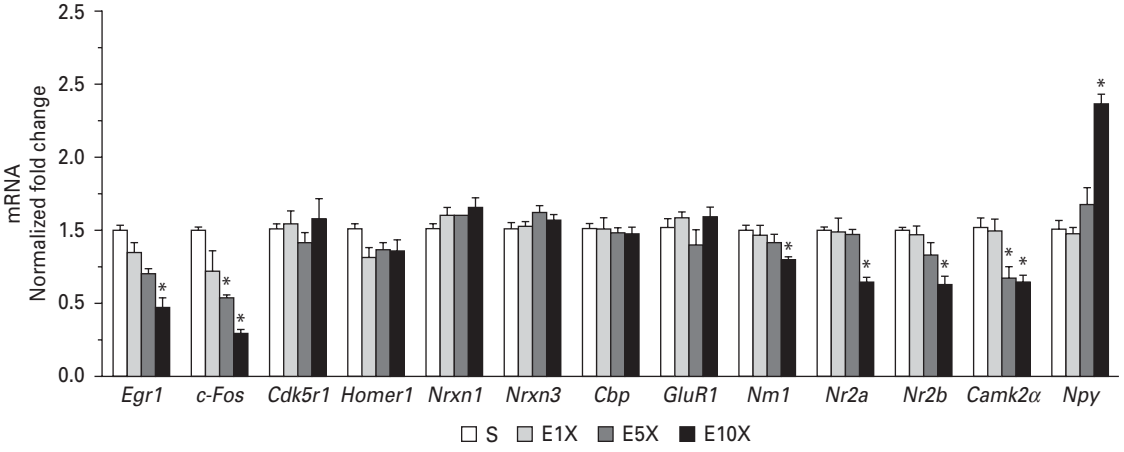

Fig. 3. Effects of ECS treatments on mRNA expression of HDAC2-regulated genes in the rat frontal cortex. mRNA expression levels of genes regulated by HDAC2, including *Egr1, c-Fos*, cyclin-dependent kinase 5, regulatory subunit 1 (*Cdk5r1*), *Homer1*, neurexin1 (*Nrxn1*), neurexin3 (*Nrxn3*), CREB binding protein (*Cbp*), glutamate receptor, ionotropic, AMPA 1 (*Glur1*), *Nrn1, Nr2a, Nr2b*, and *Camk2α* and of *Npy* were analyzed with qRT-PCR, and values relative to the sham control (S) are presented. mRNA levels of *c-Fos* and *Camk2α* were significantly reduced 24 h after repeated ECS treatments for 5 d (E5X). mRNA levels of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α* were significantly reduced 24 h after repeated ECS treatments for 10 d (E10X). E10X induced a significant increase in the mRNA level of *Npy*. Values are presented as means±S.E. *, $p<0.05$ ($n=4$–8 for each treatment group).

performed. The density of HDAC2-positive neurons was increased in the prefrontal cortex after E10X (Fig. 2*a*). HDAC2-positive cells were increased significantly in E10X-treated animals in the prefrontal (Fr; $t=2.917$, $p=0.027$), cingulate (Cg; $t=3.385$, $p=0.015$), insular (AI; $t=3.611$, $p=0.011$), and orbital (LO and VLO; $t=3.770$, $p=0.009$) cortices (Paxinos and Watson, 1998) (Fig. 2*b*).

Immunofluorescence analysis was performed. Immunoreactivity of HDAC2 was co-localized with Neu-N, a specific neuronal protein (Mullen et al., 1992), but not with GFAP, a marker for glial cells (Raff et al., 1979). Additionally, HDAC2 immunoreactivity was co-localized with DAPI reactivity, a fluorescence stain labeling cell nuclei through binding to DNA (Kubista et al., 1987) (Fig. 2*c*). E10X increased the HDAC2 immunoreactivity in the nucleus of the neurons in rat cortical regions, including the prefrontal, cingulate, orbital, and insular cortices.

Repeated ECS treatments reduced *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α* mRNA expression in the rat frontal cortex.

The mRNA expression levels of genes reportedly regulated by HDAC2 (Guan et al., 2009) including *Egr1, c-Fos*, cyclin-dependent kinase 5, regulatory subunit 1 (*Cdk5r1*), *Homer1*, neurexin1 (*Nrxn1*), neurexin3 (*Nrxn3*), CREB binding protein (*Cbp*), glutamate receptor, ionotropic, AMPA 1 (*Glur1*), *Nrn1, Nr2a, Nr2b*, and *Camk2α* were investigated by qRT-PCR. We selected 11 genes that Guan et al. (2009) reported were implicated in synaptic remodeling/plasticity or were regulated by neuronal activity among genes with a more selective association with HDAC2 than with HDAC1. As a control experiment, mRNA expression of *Npy*, which is known to be induced by repeated ECS treatments (Mikkelsen et al., 1994), was also examined. The mRNA levels of *Egr1* ($F=7.774$, $p=0.001$), *c-Fos* ($F=10.609$, $p<0.001$), *Nrn1* ($F=8.897$, $p<0.001$), *Nr2a* ($F=10.708$, $p<0.001$), *Nr2b* ($F=6.428$, $p=0.003$), *Camk2α* ($F=8.891$, $p=0.001$), and *Npy* ($F=28.819$, $p<0.001$) were significantly modified after ECS treatments. The mRNA expression levels of *c-Fos* ($p=0.029$) and *Camk2α* ($p=0.006$) were reduced by E5X. The mRNA level of *Egr1* ($p<0.001$) was reduced in the E10X group, as reported previously (Park et al., 2011). The mRNA levels of *c-Fos* ($p<0.001$), *Nrn1* ($p<0.001$), *Nr2a* ($p<0.001$), *Nr2b* ($p<0.001$), and *Camk2α* ($p=0.001$) were reduced by E10X. The *Npy* mRNA level was significantly increased by E10X ($p<0.001$). The mRNA levels of *Cdk5r1, Homer1, Nrxn1, Nrxn3, Cbp*, and *Glur1* were not significantly altered (Fig. 3).

Reduced histone acetylation in promoter regions of HDAC2-regulated genes

We investigated histone acetylation in the promoter regions of the genes showing reduced expression with E10X using a ChIP assay followed by qRT-PCR. The acetylation of histone H3 ($t=4.540$, $p=0.002$) and H4 ($t=2.384$, $p=0.044$) was reduced at the *Egr1* promoter. At the promoter regions of *c-Fos, Nrn1, Nr2a*, and *Nr2b*, histone H4 acetylation, but not H3 acetylation, was reduced significantly ($t=8.849$, $p<0.001$; $t=4.158$, $p=0.006$; $t=11.680$, $p<0.001$; $t=9.615$, $p<0.001$, respectively). At the *Camk*2α promoter region, the histone H3 acetylation level was decreased ($t=5.092$, $p=0.002$). Acetylation of H3 and H4 at the promoter region of *Npy* was increased significantly ($t=4.127$, $p=0.005$; $t=6.965$, $p<0.001$; Fig. 4*a*).

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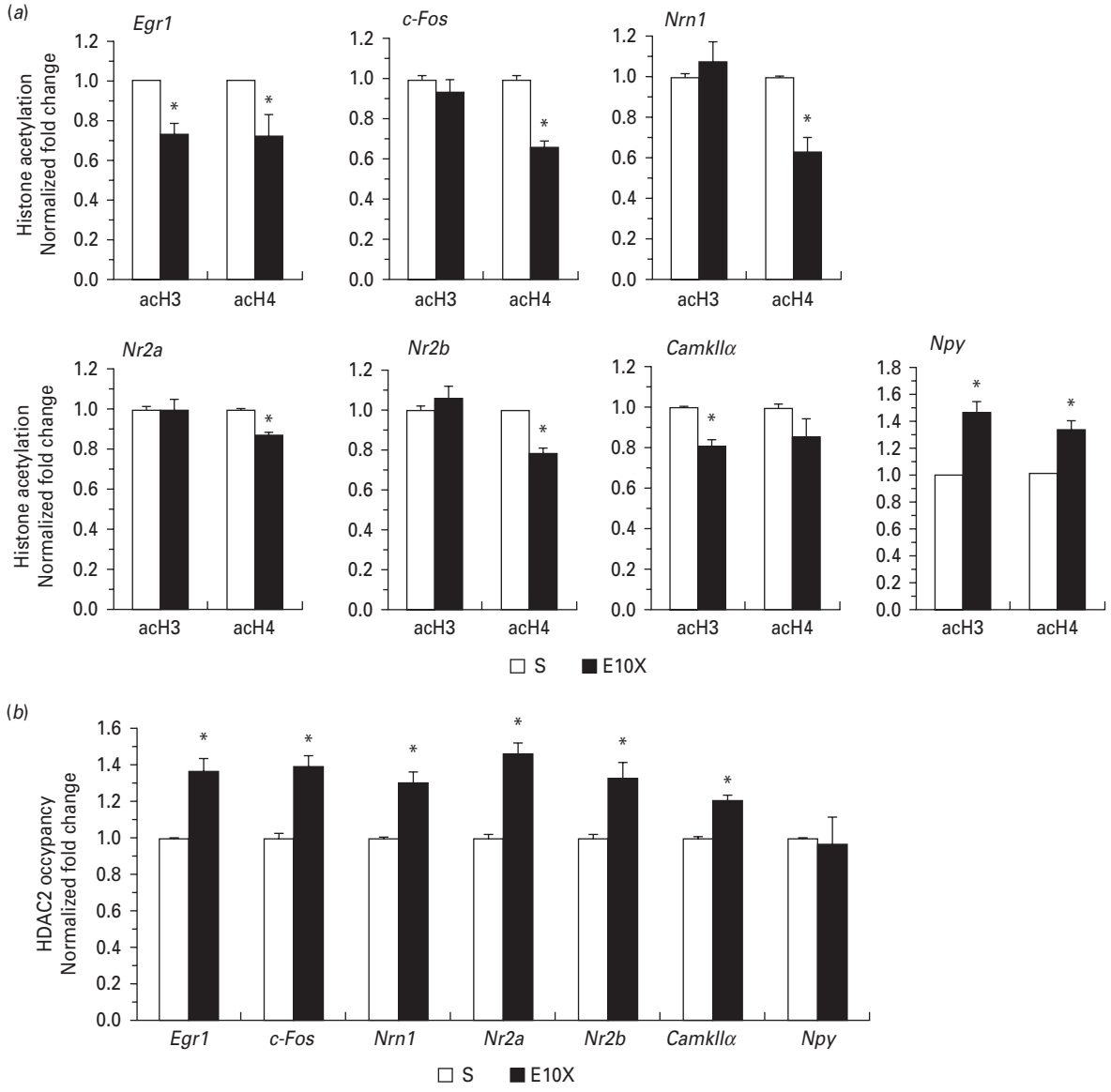

Fig. 4. Reduced histone acetylation and increased HDAC2 occupancy in the promoter regions of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α* in the rat frontal cortex after repeated ECS treatments. (*a*) Histone acetylation at the promoter regions of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b, Camk2α*, and *Npy* was analyzed with a ChIP assay followed by qRT-PCR, and values relative to the sham control (S) are presented. Repeated ECS treatments for 10 d (E10X) reduced H3 acetylation of *Egr1* and *Camk2α* genes and H4 acetylation of *Egr1, c-Fos, Nrn1, Nr2a*, and *Nr2b* genes in the rat frontal cortex. In contrast, acetylation of H3 and H4 was increased in the *Npy* gene. (*b*) Binding of HDAC2 at the promoter regions of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b, Camk2α*, and *Npy* was analyzed with ChIP assay followed by qRT-PCR, and values relative to the sham control (S) are presented. E10X induced increased occupancy of HDAC2 at the promoter regions of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α* genes. *, $p<0.05$ ($n=4$ for each treatment group).

Increased binding of HDAC2 to the promoter regions of ECS-reduced genes in the rat frontal cortex

We investigated whether the increased expression of HDAC2 was related to the E10X-induced down-regulation of expression and histone acetylation of the genes examined. The occupancy of HDAC2 at the promoter regions of the genes was examined by ChIP followed by qRT-PCR. HDAC2 binding was increased in the promoter regions of *Egr1* ($t=3.533$, $p=0.012$), *c-Fos* ($t=5.212$, $p=0.002$), *Nrn1* ($t=3.450$, $p=0.014$), *Nr2a* ($t=4.123$, $p=0.018$), *Nr2b* ($t=5.552$, $p=0.001$), and *Camk2α* ($t=7.902$, $p<0.001$) after E10X. However, HDAC2 occupancy in the *Npy* promoter was unaffected by E10X (Fig. 4*b*).

Treatment with sodium butyrate, a HDAC inhibitor, restores ECS-induced down-regulation of HDAC2-related gene expression

To investigate whether HDAC2 enrichment at the promoter regions of genes down-regulated by ECS was functionally significant, the effect of NaBu, which predominantly inhibits class I HDACs (Haggarty and

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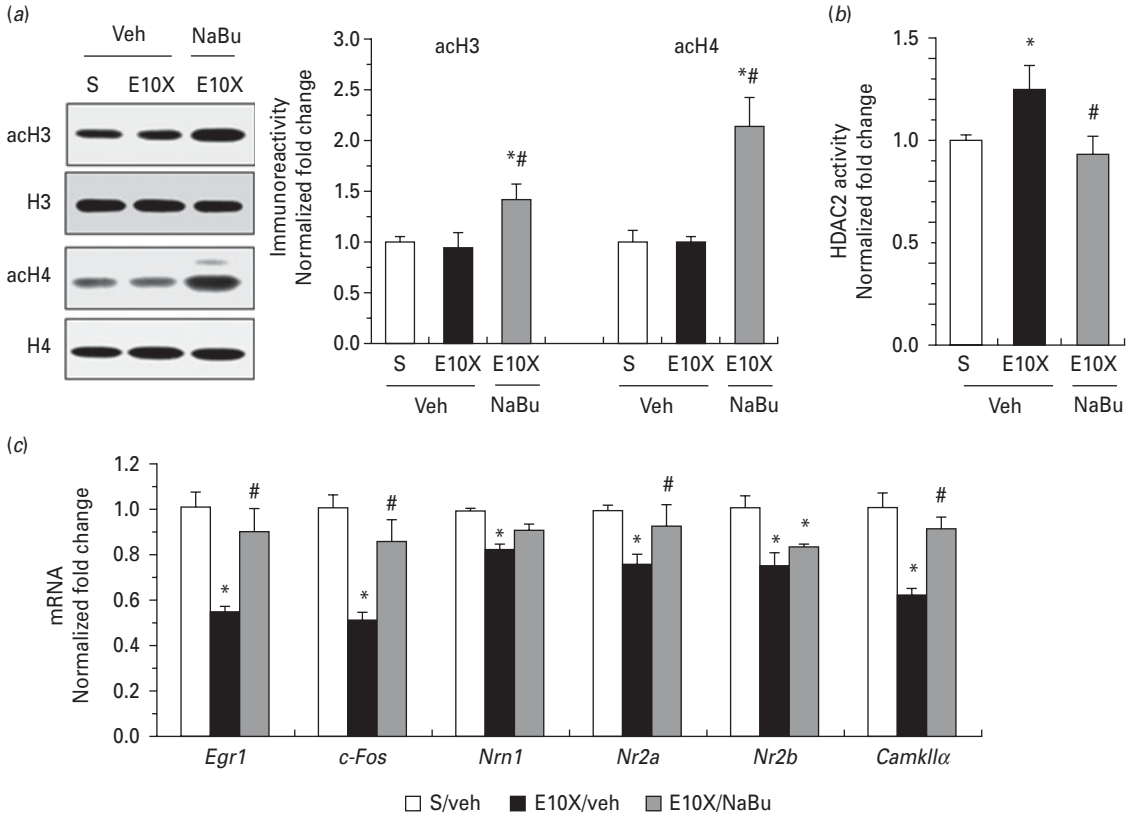

Fig. 5. Administration of sodium butyrate restores ECS-induced downregulation of gene expression in the rat frontal cortex. (*a*) Global histone acetylation levels of H3 and H4 in the rat frontal cortex were analyzed after 5 d of once-daily i.p. injections of NaBu (1200 mg/kg), and quantified immunoblot values are presented. NaBu treatment induced a significant increase in the acetylation of H3 and H4 in the rat frontal cortex. (*b*) HDAC2 activity in the rat frontal cortex was analyzed, and relative activity values are presented. NaBu treatment significantly reduced the E10X-induced increase in HDAC2 activity. (*c*) mRNA expression levels of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α* after repeated ECS treatment and NaBu were analyzed with qRT-PCR, and values relative to the sham/vehicle control (S/veh) are presented. mRNA levels of *Egr1, c-Fos, Nr2a*, and *Camk2α* in the E10X/NaBu group were significantly higher than those in the E10X/veh group, and the mRNA level of *Nrn1* in the E10X/NaBu group was not significantly lower than that in the S/veh group. *, $p<0.05$, compared with S/veh group; #, $p<0.05$, compared with E10X/veh group ($n=5$ or 6 for each treatment group).

Tsai, 2011), on the reduced expression of HDAC2 target genes was examined. NaBu was injected once per day for 5 d. First, the effect of NaBu on global histone acetylation and on HDAC2 activity in the rat frontal cortex was examined to confirm the efficacy of the NaBu injection. Systemic injection of NaBu significantly affected the acetylation level of H3 ($F=5.482$, $p=0.038$) and H4 ($F=6.269$, $p=0.020$) in the rat frontal cortex. The acetylation levels of H3 and H4 in the E10X/NaBu group were significantly higher than were those in the E10X/veh group ($p=0.022$ and 0.038, respectively) and in the S/veh group ($p=0.018$ and 0.017, respectively; Fig. 5*a*). In addition, systemic injection of NaBu significantly affected HDAC2 activity in the rat frontal cortex ($F=4.940$, $p=0.024$). HDAC2 activity was significantly greater in the E10X/veh group than in the S/veh group ($p=0.025$), while HDAC2 activity was significantly lower in the E10X/NaBu group than in the E10X/veh group ($p=0.011$) (Fig. 5*b*). These findings indicate that HDAC2 activity was increased in the rat frontal cortex by E10X, which was efficiently inhibited by systemic NaBu treatments. Reduced mRNA expression of *Egr1, c-Fos, Nrn1, Nr2a*, and *Camk2α* by E10X (E10X/veh) compared with the sham control group (S/veh) was not evident with NaBu pretreatment before ECS (E10X/NaBu) in the rat frontal cortex. mRNA expression levels of *Egr1* ($F=7.533$, $p=0.010$), *c-Fos* ($F=9.109$, $p=0.034$), *Nr2a* ($F=12.800$, $p=0.040$), and *Camk2α* ($F=11.256$, $p=0.004$) in the E10X/NaBu group were significantly higher than in the E10X/veh group. The reduced mRNA expression of *Nr2b* due to E10X was not significantly recovered by NaBu treatment. Down-regulated gene expression, except *Nr2b*, due to E10X was demonstrated to recover with co-administration of NaBu.

Discussion

Repeated ECS treatment was demonstrated to up-regulate the expression level of HDAC2, a class I HDAC, in the nuclei of neurons in the rat frontal cortical

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regions. Expression levels of HDAC2 target genes in the brain (Guan et al., 2009) were examined. Repeated ECS treatments reduced the mRNA levels of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α*, which are major components of *N*-methyl-D-aspartate (NMDA) receptor-related signaling. In the promoter regions of these genes, the histone acetylation levels of H3 and/or H4 were decreased and HDAC2 occupancy was increased by repeated ECS treatments. Administration of NaBu, an HDAC inhibitor, recovered the reduced expression of *Egr1, c-Fos, Nrn1, Nr2a*, and *Camk2α* due to repeated ECS treatments. These findings suggest that repeated ECS treatments resulted in reduced expression of NMDA receptor signaling-related genes through histone deacetylation at the promoter regions via ECS-induced HDAC2 in the rat frontal cortex.

We previously found that repeated ECS treatments down-regulated the expression and inducibility of *Egr1* and *c-Fos* in the rat frontal cortex (Park et al., 2011). In this study, histone acetylation at the promoters of *Egr1* and *c-Fos* was demonstrated to be reduced by E10X. These findings could provide an explanation for the reduced expression and inducibility of the IEGs, *c-Fos* and *Egr1*, by E10X, insofar as histone deacetylation-related chromatin condensation could repress stimulus-responsive gene transcription. Reduced expression and histone deacetylation of *c-Fos* in the rat hippocampus with repeated ECS treatments has been reported previously (Tsankova et al., 2004), which is consistent with the present findings in the frontal cortex.

Among class I HDACs, HDAC2 was increased in the rat frontal cortex by repeated ECS treatments. Histone acetylation of *Egr1* was reduced, and the occupancy of HDAC2 in the *Egr1* gene promoter was increased in the E10X group. These results are consistent with a previous report of a decreased number of Egr1-positive neurons in HDAC2 over-expressing mice, and the opposite observation in *Hdac2* knockout mice (Guan et al., 2009). mRNA levels of *c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α*, target genes of HDAC2 in the brain (Guan et al., 2009), were also reduced, accompanied by histone deacetylation and increased HDAC2 occupancy at their promoters, by E10X. The reduced mRNA levels of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α* were not evident after E1X, when the HDAC2 level was not increased. These findings suggest that HDAC2 could be involved in the down-regulation of these genes in response to repeated ECS treatments.

Among the genes showing reduced expression with E10X, NaBu treatment restored the mRNA expression levels of *Egr1, c-Fos, Nrn1, Nr2a*, and *CaMKIIα*. Among four major classes of HDAC inhibitors, NaBu belongs to the first class of HDAC inhibitors, which predominantly inhibit class I HDACs (Haggarty and Tsai, 2011). Although NaBu also inhibits class IIa HDACs (Carew et al., 2008), class IIa HDACs are likely to act through non-histone substrates, because they have low basal HDAC activity (Lahm et al., 2007). Decreased histone acetylation and increased binding of HDAC2 at the promoters of the genes showing reduced expression suggest a possible role for HDAC2 in down-regulating the expression of the genes. The decreased mRNA expression of the genes and the increased HDAC2 activity by E10X were reversed by NaBu treatment. These findings suggest that involvement of HDAC2 may play an important role in the ECS-induced down-regulation of gene expression. However, reduced *Nr2b* expression was not recovered by NaBu treatment. Other epigenetic mechanisms, including DNA methylation or other histone modifications, are presumably involved, but this requires further clarification. For example, ECS induces an increase in DNA methylation of the *Arc* gene promoter, which could supress *Arc* expression (Dyrvig et al., 2012). In addition, transcriptional activity can be regulated by epigenetic changes in the distal promoter region (Coskun et al., 2012). In the current study, we only examined the proximal promoter regions of the genes. Further studies to examine the possible involvement of other epigenetic mechanisms and distal promoters are required for clarification.

HDAC2 affects synaptic transmission in neurons and is involved in the molecular machinery regulating the differentiation and integration of neurons (Akhtar et al., 2009; Montgomery et al., 2009). HDAC2 is involved in modulating synaptic plasticity and long-lasting changes in neural circuits through binding to synaptic plasticity-related genes (Guan et al., 2009). Repeated ECS treatments down-regulated the expression levels of *Egr1, c-Fos, Nrn1, Nr2a*, and *Camk2α*, which are involved in NMDA receptor-mediated synaptic plasticity. *Nr2a* and *Nr2b* genes encode subunits of ionotropic NMDA receptor (Goebel and Poosch, 1999). CaMKIIα protein is abundantly expressed in the postsynaptic density, and CaMKIIα, bound to NMDA receptors, plays important roles in mediating long-term potentiation (LTP) (Bayer et al., 2001; Hudmon and Schulman, 2002; Lisman et al., 2002; Sanhueza et al., 2011). *Nrn1* encodes neuritin 1 protein, which plays critical roles in dendritic outgrowth, maturation, and axonal regeneration (Naeve et al., 1997; Nedivi et al., 1998; Fujino et al., 2008; Karamoysoyli et al., 2008). *Nrn1* expression can be induced by neural activity and neurotrophins (Naeve et al., 1997; Nedivi et al., 1998). *Nrn1* is induced by calcium influx through NMDA receptors, which is involved in an activity-regulated transcriptional program that induces long-term changes in synaptic connections (Fujino et al., 2003, 2008). *Egr1* and *c-Fos*, as IEGs, are induced by changes in neural activity subsequent to NMDA receptor activation (Beckmann and Wilce, 1997; Guzowski et al., 2001; Mokin and Keifer, 2005). The reduced expression of NMDA receptor-related genes by repeated ECS treatments might be related to a reduced response to neuronal excitability.

The clinical effects of ECT are exerted, at least partly, by reduced neural excitability and modified synaptic

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plasticity (Kato, 2009). Repeated ECS treatments induce anti-convulsive properties, which have been suggested to play important roles in the therapeutic mechanism(s) of ECT (Caplan, 1946; Sackeim et al., 1983; Post et al., 1984; Sackeim, 1999). Down-regulation of genes in the NMDA receptor signaling pathway by ECS-induced HDAC2 might be related to reduced neural excitability and the anti-convulsive actions of repeated ECS treatments. A significant increase in HDAC2 expression in an experimental animal model of temporal lobe epilepsy (TLE) and in the brains of patients with TLE has been reported, and this was suggested to be an adaptive change in response to repeated seizures (Huang et al., 2012b). HDAC2 was increased after repeated ECS treatments, but not after a single treatment. Taken together, these results indicate that increased HDAC2 could be involved in the chronic adaptive changes induced by repeated ECS treatments. Transcription factors regulating the expression of *Hdac2* include β-catenin, avian myelocytomatosis viral oncogene homolog (Myc), activator protein 2 (AP2), and lymophoid enhancer factor 4 (LEF4) (Zhu et al., 2004; Zimmermann et al., 2007; Kramer, 2009). Recently, the involvement of serotonin 5-HT_{2A} receptor in the up-regulation of *Hdac2* expression in mouse frontal cortex was reported (Kurita et al., 2012). Further investigations are required to understand the roles and implications of HDAC2 induction in the mechanisms of action of ECS.

Antidepressants and antipsychotics have been reported to induce changes in the expression of HDACs in the brain. Repeated treatment with fluoxetine was reported to increase *Hdac2* mRNA expression in the rat striatum and hippocampus (Cassel et al., 2006). Antidepressant treatments, including clomipramine, escitalopram, duloxetine, and mirtazapine, result in region-specific induction of various HDAC isoforms, including HDAC2, HDAC3, and HDAC5, in the brains of mice (Ookubo et al., 2013). In the report of Kurita et al. (2012), the atypical antipsychotic agent clozapine increased HDAC2 expression in the mouse frontal cortex and in the post-mortem human brain of schizophrenic subjects. HDAC2 reduces transcription of metabotropic glutamate 2 receptor (mGlu2), which has been demonstrated to limit the therapeutic effects of antipsychotics. Further clarification is required to determine whether repeated ECS treatment-induced HDAC2 facilitates or limits the therapeutic effects of ECS as an adaptive response to repeated seizures.

In summary, repeated ECS treatments induced HDAC2, one of the class I HDACs, in the rat frontal cortex. Transcription and promoter histone acetylation of *Egr1, c-Fos, Nrn1, Nr2a, Nr2b*, and *Camk2α* genes were reduced, and HDAC2 occupancy on the promoters of these genes was increased. Moreover, administration of the HDAC inhibitor NaBu restored the repeated ECS-induced reduced gene expression. These findings suggest that repeated ECS treatment could down-regulate the genes involved in the NMDA receptor signaling pathway through HDAC2 induction. Further studies are required to understand the role of HDAC2 induction in the therapeutic and adverse effects of ECS.

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Statement of Interest

There is no actual or potential conflict of interest in relation to this article. Y.M. Ahn declares that he has received research grants from, or served as a lecturer for, Janssen, Otsuka, and Lundbeck. Y.S. Kim declares that he has received grants, research support, and/or honoraria from Norvatis, Janssen, Otsuka, and AstraZeneca. The present study was not related to any of these professional relationships.

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