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Original Article

ORIGINAL ARTICLE

Developmental Alcohol Exposure Impairs Activity-Dependent S-Nitrosylation of NDEL1 for Neuronal Maturation

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Abstract

Neuronal nitric oxide synthase is involved in diverse signaling cascades that regulate neuronal development and functions via S-Nitrosylation-mediated mechanism or the soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway activated by nitric oxide. Although it has been studied extensively in vitro and in invertebrate animals, effects on mammalian brain development and underlying mechanisms remain poorly understood. Here we report that genetic deletion of "Nos1" disrupts dendritic development, whereas pharmacological inhibition of the sGC/cGMP pathway does not alter dendritic growth during cerebral cortex development. Instead, nuclear distribution element-like (NDEL1), a protein that regulates dendritic development, is specifically S-nitrosylated at cysteine 203, thereby accelerating dendritic arborization. This post-translational modification is enhanced by *N*-methyl-D-aspartate receptor-mediated neuronal activity, the main regulator of dendritic formation. Notably, we found that disruption of S-Nitrosylation of NDEL1 mediates impaired dendritic maturation caused by developmental alcohol exposure, a model of developmental brain abnormalities resulting from maternal alcohol use. These results highlight S-Nitrosylation as a key activity-dependent mechanism underlying neonatal brain maturation and suggest that reduction of S-Nitrosylation of NDEL1 acts as a pathological factor mediating neurodevelopmental abnormalities caused by maternal alcohol exposure.

Key words: S-Nitrosylation, NDEL1, dendritic development, neuronal activity, fetal alcohol spectrum disorder

Introduction

Nitric oxide (NO), an essential gaseous neurotransmitter involved in synaptic function, is produced by neuronal nitric oxide synthase (nNOS: Gene name is Nos1) in the central nervous system (CNS) (Lipton et al. 1994; Shahani and Sawa 2011). nNOS is highly expressed in the cerebral cortex during brain development (Bredt and Snyder 1994; Eliasson et al. 1997; Kwan et al. 2012). Consistently, NO is abundantly produced in neonatal cerebral cortex (Imura et al. 2005). Nonetheless, nNOS-mediated mechanisms underlying formation of neuronal architecture during the neonatal period remain to be investigated.

nNOS-mediated NO production activates 2 major downstream mechanisms, namely the soluble guanylate cyclase (sGC)/cyclic guanosine monophosphate (cGMP) pathway and the S-Nitrosylation cascade (Garthwaite et al. 1988; Nakamura et al. 2013). NO stimulates sGC to generate cGMP, and impact various physiological processes, such as synaptic plasticity, neurotransmitter release, and regulation of ion channels in the CNS (Denninger and Marletta 1999; Serulle et al. 2007; Shelly et al. 2010; Sunico et al. 2010). S-Nitrosylation is another major modality of NO signaling involving a post-translational modification of cysteine residues in proteins, resulting in conformational changes that regulate functional activity (Lei et al. 1992; Stamler et al. 1992; Jaffrey et al. 2001; Nakamura et al. 2013). Multiple proteins have been identified as targets of S-Nitrosylation which exerts diverse effects on various neuronal processes including synaptic function (Fang et al. 2000; Hara et al. 2005; Uehara et al. 2006; Nott et al. 2008; Zhang et al. 2010; Ho et al. 2011; Qu et al. 2011; Nakamura et al. 2013; Selvakumar et al. 2013). These nNOS-mediated mechanisms have been investigated extensively in cell and invertebrate models, as well as in mouse brains during the prenatal period (Cramer et al. 1996; Gibbs and Truman 1998; Inglis et al. 1998; Hara et al. 2005; Uehara et al. 2006; Nikonenko et al. 2008; Lee et al. 2009; Shelly et al. 2010; Selvakumar et al. 2013), whereas nNOS signaling in neonatal brain development has not been characterized.

Reduction of NOS activity has been highlighted in developing brains in mice treated with ethanol during the prenatal period, a model of fetal alcohol spectrum disorders (FASDs) caused by maternal alcohol consumption (Kimura et al. 1996; Kimura and Brien 1998; Chokroborty-Hoque et al. 2014). The detrimental effect of alcohol exposure in rodent developmental periods equivalent to the third trimester of human pregnancy (Klintsova et al. 2012), when dendritic development and synaptogenesis occur (Huttenlocher and Dabholkar 1997), has also been recently investigated in rodent models subjected to neonatal alcohol exposure (Granato et al. 2003; Livy et al. 2003; Mooney and Napper 2005; Hamilton et al. 2010; Diaz et al. 2014). Neonatal ethanol exposure induces substantial neuronal loss during brain development in nNOS KO mice, compared with wild type (WT) mice (Bonthius et al. 2002; de Licona et al. 2009). Nevertheless, nNOS-mediated mechanisms underlying the effect of alcohol exposure on neonatal brain development are poorly understood.

Here, we determine that nNOS-mediated S-Nitrosylation is a crucial regulatory mechanism for dendritic development, a critical basis for synaptic integration coordinated by neuronal activity (Lipton et al. 1994; Cline 2001; Konur and Ghosh 2005; Parrish et al. 2007). Surprisingly, multiple inhibitors of the sGC/cGMP pathway during the neonatal period have no robust detrimental effects on dendritic growth. In contrast, nuclear distribution element-like (NDEL1), a molecule involved in multiple developmental cellular processes (Niethammer et al. 2000; Sasaki et al. 2000; Bradshaw et al. 2011), is specifically S-nitrosylated at cysteine 203, thereby accelerating dendritic arborization. This post-translational modification is enhanced by N-methyl-D-aspartate (NMDA) receptor-mediated neuronal activity, indicating that activity-dependent S-nitrosylation of NDEL1 is required for proper dendritic development. Of note, dendritic impairment caused by neonatal ethanol exposure was normalized by up-regulation of NDEL1 S-Nitrosylation, suggesting that S-Nitrosylation of NDEL1 is a critical mechanism for alcohol-induced developmental brain abnormalities.

Materials and Methods

Experimental procedures for in utero electroporation, dendrite analysis, biotin-switch assay, and alcohol administration are described here. All other detailed methods can be found under Supplementary Materials and Methods.

In utero Electroporation

In utero electroporation, specifically targeting the prefrontal cortex (PFC) was performed according to our previously published protocols (Niwa et al. 2010; Saito et al. 2016). C57/BL6 mice (Charles River) and heterozygous conditional "Ndel1" deletion (NDEL1 CKO) mice were used in this study. Inducible gene manipulation was conducted following published methods with minor modifications (Matsuda and Cepko 2007; Saito et al. 2016). Briefly, for genetic deletion of NDEL1 and green fluorescent protein (GFP)-labeling in post-migratory neurons in the PFC in NDEL1 CKO mice, CALNL-GFP and CAG-ER^{T2}CreER^{T2} were injected into the lateral ventricle and co-electroporated into the ventricular zone of C57/BL6 at E15. Pups were then given 4-hydroxytamoxifen (4-OHT) (2 mg per 100 g body weight) via intraperitoneal injection at P2 for inducible gene expression or genetic deletion of Ndel1, as previously described (Matsuda and Cepko 2007). Inducible short hairpin RNA (shRNA) for NDEL1 (1 μg/μL) and CALNL-GFP (1 μg/μL) together with CAG-ER^{T2}CreER^{T2} (0.5 μg/μL) (molar ratio, approximately 2:2:1) were co-electroporated for inducible knockdown of NDEL1. Inducible expression constructs of human WT and mutant NDEL1 (CALNL-NDEL1-WT, CALNL-NDEL1-C203S, or CALNL-NDEL1-C203W) (1 μg/μl) were co-electroporated to test functional complementation. Pups were then given 4-OHT as described above.

Dendrite Analysis

Dendrites were analyzed by morphological assessment of fluorescently labeled pyramidal neurons in medial PFC regions, according to modifications of previously published protocols (Saito et al. 2016). Images were collected as Z stacks with a 40x oil-immersion objective lens of a confocal microscope (LSM 700, Zeiss). A scan speed of 9 was used, with images taken at 1.0 μm interval of Z-sections, in a pixel resolution of 1024 × 1024. Parameters were homologous for all scans. A region of interest was randomly selected (800 μm wide × 800 μm high) in the mPFC where GFP-positive cells were located. Z stacks were compressed, and resulting images were analyzed with NeuronJ, a "plug-in software for ImageJ (http://www.imagescience.org/meijering/software/neuronj/). To trace dendrites in a single GFP-positive neuron, the dendrites originating from other adjacent GFP-positive neurons were excluded based on observation of uncompressed Z-scan images. Primary, secondary, and tertiary branches of both apical and basal dendrites were counted under each condition. Only neurons with typical morphological characteristics of classic

pyramidal neurons (single thick axon arising from the apical side of the soma with multiple basal dendrites and extending vertically to layer I) were analyzed. Five independent brain slices were analyzed in a blinded manner. Dendritic branching in primary cortical neurons at 7 days in vitro (7 DIV) was analyzed by a previously published method (Saito et al. 2016).

Biotin-Switch Assay

The biotin-switch assay was performed by minor modifications of our previously published protocols (Jaffrey et al. 2001; Selvakumar et al. 2013). Briefly, frontal cortex extracts were lysed and homogenized in HEN buffer (250 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, pH 7.7, and protease inhibitors) with 1% Triton X-100, followed by passage through a syringe with a 26-gauge needle, 5 times. Protein lysates from cell cultures were prepared by using the same method without homogenization. The resulting lysates were incubated for 20 min at 4 °C, followed by centrifugation at 15 000 rpm. Supernatants from lysed protein samples were quantified by BCA protein assay kit (Thermo Fisher scientific 23 225), and 80 μg of proteins were used in each assay. The samples were treated with 0.3% methyl methanethiosulfonate (MMTS) and 2.5% sodium dodecyl sulfate (SDS) at 50 °C for 20 min. Residual MMTS and SDS were removed by spin down 3 times through Micro Bio-SpinTM 6 Columns (Bio-Rad 732–6221). To reduce nitrosothioles to reform the thiol selectively, the samples were incubated with 50 mM ascorbate for 90 min at room temperature, and labeled with biotin-HPDP (0.4 mM N-[6-(Biotinamido) hexyl]-3′-(2′pyridyldithio)propionamide) (Soltec Ventures B106). The biotinylated proteins were collected by High Capacity NeutrAvidin Agarose (Thermo Fisher scientific 29 202) beads with rotation overnight at 4 °C. After washing 3 times with HEN buffer, the biotinylated proteins were dissolved from the beads with NuPAGE LDS sample buffer (Life technologies NP0007) and denatured at 95 °C for 10 min. Biotinylated NDEL1 was detected by western blotting with a mouse monoclonal antibody against NDEL1 (abcam ab119005).

Alcohol Administration

The mice were intraperitoneally injected with phosphate-buffered saline (PBS) or 25% ethanol in PBS (final 5.0 g/kg weight) once a day from P4 to P7, equivalent to the human third trimester (Livy et al. 2003; Mooney and Napper 2005; Hamilton et al. 2010).

Results

Genetic Deletion of Nos1, but Not Blockade of sGC/cGMP Pathway, Impairs Dendritic Development in the PFC

To investigate whether nNOS is required for proper dendritic development in the PFC, dendritic structures of pyramidal neurons were examined in homozygous “nitric oxide synthase 1 (Nos1)” gene knockout (nNOS KO) mice and WT littermates in which pyramidal neurons of layer II/III were labeled with GFP by in utero electroporation (Niwa et al. 2010; Saito et al. 2016). GFP expression plasmid was electroporated into the medial PFC at embryonic day 15 (E15), and dendritic structures were analyzed at postnatal day 7 (P7), a period of robust dendritic growth (Saito et al. 2016). We confirmed that nNOS is expressed in the GFP-labeled pyramidal neurons collected by Fluorescence-Activated Cell Sorting (FACS) from the dissociated PFC at P7 (Supplementary Fig. 1). We observed that the total numbers of apical and basal dendrites, as well as the number of tertiary branches, both apical and basal, were significantly decreased in GFP-labeled pyramidal neurons of nNOS KO mice (Fig. 1*A*,*B*). Previous studies have shown a possible involvement of the NO-mediated sGC/cGMP pathway in the refinement of axonal projections and dendrite and spine morphologies under in vitro conditions and in invertebrate models (Cramer et al. 1996; Hindley et al. 1997; Gibbs and Truman 1998; Nikonenko et al. 2008; Stern and Bicker 2008; Lee et al. 2009; Shelly et al. 2010). To examine whether the sGC/cGMP pathways play a major role in dendritic development in vivo, the wild-type mouse pups labeled with GFP in pyramidal neurons in the layer II/III were given 1H-[1,2,4]oxadiazolo [4,3,-a] quinoxalin-1-one (ODQ), a selective guanylate cyclase inhibitor, via daily intraperitoneal injection starting from P3, after which dendrite formation in the medial PFC was assessed at P7. Although ODQ treatment reduced cGMP activity in the PFC (Fig. 1*C*), no significant change in dendritic development was observed under these conditions (Fig. 1*D*,*E*, Supplementary Fig. 2). Consistently, when pups were treated daily with KT5823, an inhibitor of protein kinase G, which is a cGMP-dependent downstream effector, dendritic structure remained intact (Fig. 1*D*,*E*, Supplementary Fig. 2). These results indicate that the sGC/cGMP pathway does not play a central role in dendritic development in the developing medial PFC.

NDEL1 Is S-nitrosylated in Developing Brain

We next explored S-Nitrosylation as a potential nNOS-mediated mechanism underlying regulation of dendrite formation. Previous studies demonstrated that S-Nitrosylation of cyclin dependent kinase 5 (CDK5) and histone deacetylase 2 (HDAC2) are required for neuronal developmental process, such as neuronal migration (Nott et al. 2008, 2013; Zhang et al. 2010). Thus, we wondered whether these molecules may also be S-nitrosylated during the neonatal period, and may be critical for dendritic development. To test this idea, protein lysates from the frontal cortex of wild-type littermates were assessed at P7 through a biotin switch assay, a method that we have previously established for labeling S-nitrosylated proteins with a biotin moiety on S-nitrosylated cysteines (Jaffrey et al. 2001). Although treatment with S-nitrosoglutathione (GSNO), an NO donor, increased S-Nitrosylation of CDK5 and HDAC2, neither endogenously S-nitrosylated CDK5 nor HDAC2 were detected by biotin switch assay during the neonatal period (Fig. 2*A*). Our previous work suggested that nNOS may interact with NDEL1, another molecule highly expressed in the developing cerebral cortex and involved in multiple developmental processes, including neurite outgrowth (Niethammer et al. 2000; Sasaki et al. 2000; Kamiya et al. 2006; Zoubovsky et al. 2011). Given that several nNOS binding proteins have been identified as S-nitrosylated proteins (Fang et al. 2000; Ho et al. 2011; Nakamura et al. 2013), we examined whether nNOS and NDEL1 interact to regulate dendritic development via S-Nitrosylation of NDEL1. Co-immunoprecipitation experiments confirmed protein interaction between endogenous nNOS and NDEL1 in developing cortical neurons at 7 DIV (Fig. 2*B*). To test whether NDEL1 is S-nitrosylated in the developing frontal cortex, protein lysates from the frontal cortex of nNOS KO mice and WT littermates were assessed. Importantly, NDEL1 was S-nitrosylated in WT littermates, but not in nNOS KO mice, suggesting that NDEL1 is S-nitrosylated by NO produced specifically by nNOS, but not by other NO synthases, such as endothelial NOS or inducible NOS in the developing frontal cortex (Fig. 2*C*).

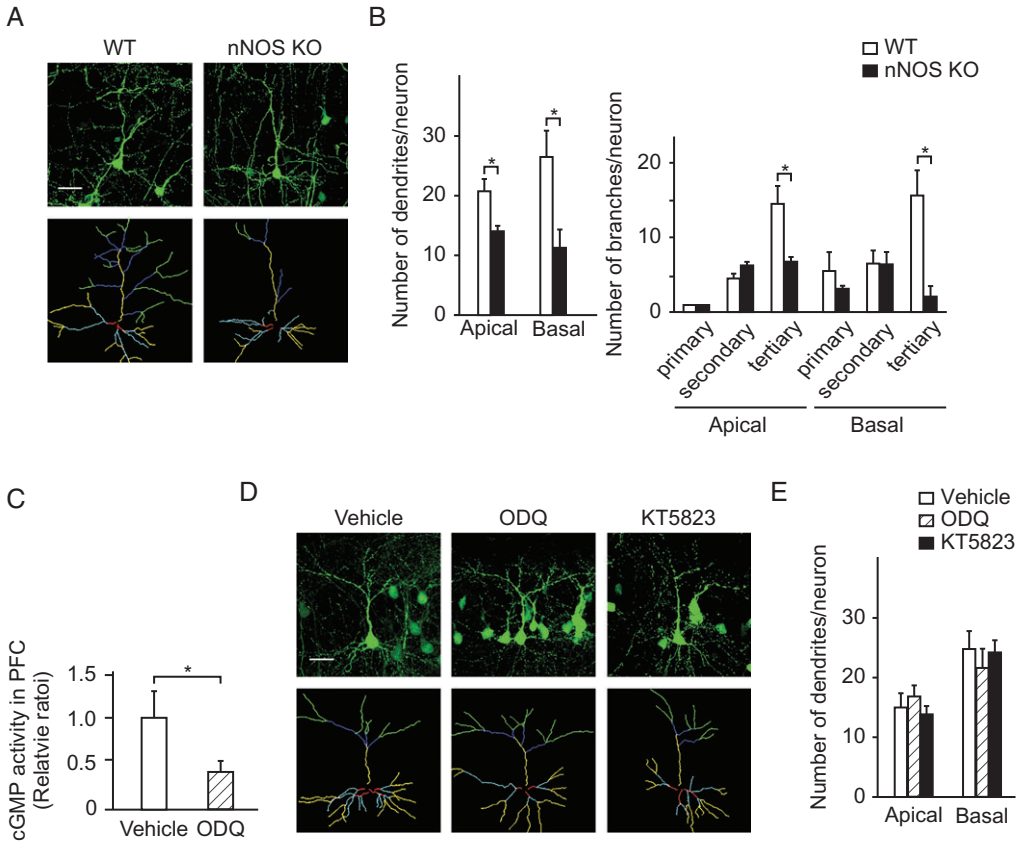

Figure 1. Genetic deletion of Nos1, but not inhibitors of the sGC/cGMP pathway, causes deficits in dendritic development in the PFC. (*A* and *B*) Dendrites of GFP-labeled pyramidal neurons in the medial PFC were analyzed in homozygous Nos1 gene knockout (nNOS KO) mice and WT littermates at postnatal day 7 (P7). Reduction in that the total numbers of apical and basal dendrites ($P = 0.0240$ and $P = 0.0295$, respectively), as well as the number of tertiary branches, both apical and basal, was observed in nNOS KO mice ($P = 0.0204$ and $P = 0.0184$, respectively). Scale bars, 25 µm. (*C*) cGMP activity was measured by immunoassay 3 h after ODQ treatment. The cGMP activity was significantly decreased in the PFC in the ODQ-treated mice compared with the vehicle-treated mice ($n = 3$). (*D* and *E*) Dendrites of GFP-labeled pyramidal neurons in the medial PFC were analyzed in the electroporated mice at P7 treated daily with ODQ (2 mg/kg body weight) or KT5823 (20 µg/kg body weight) via intraperitoneal injection starting at P3. No effect of either ODQ or KT5823 on apical and basal dendritic growth was observed under these conditions ($F(2,12) = 0.07266$, $P = 0.9303$ and $F(2,12) = 1.342$, $P = 0.2978$, respectively). Scale bars, 25 µm. (*A*, *B*, *D*, and *E*) Traced dendrites were categorized as primary apical and basal, secondary apical, and basal dendrites, as well as tertiary apical and basal dendrites, respectively. Only neurons with typical morphological characteristics of classic pyramidal neurons (single thick axon arising from the apical side of the soma with multiple basal dendrites and extending vertically to layer I) were analyzed. *$P < 0.05$ determined by Student's *t*-test (*B* and *C*) or one-way ANOVA with post hoc Tukey–Kramer test (*E*). Data are presented as the mean ± standard error of mean (SEM).

To examine the importance of NDEL1 in dendritic growth during the neonatal period, our mouse model with a conditional Ndel1 deletion (NDEL1CKO) (Sasaki et al. 2005) was used, because embryonic lethality had been shown in constitutive Ndel1 knockout mice (Sasaki et al. 2005). NDEL1 is involved in neural progenitor proliferation and neuronal migration in the cerebral cortex in early developmental periods (Niethammer et al. 2000; Sasaki et al. 2000, 2005). To segregate a role for NDEL1 in dendritic development, independent from the secondary effects of NDEL1 on dendrites caused by disturbed neural progenitor proliferation and neuronal migration, PFC-specific genetic deletion of Ndel1 in cortical pyramidal neurons during the neonatal period was performed by utilizing in utero electroporation with a previously developed Cre/loxP-mediated inducible gene expression system (Matsuda and Cepko 2007; Saito et al. 2016) (Supplementary Fig. 3). With this inducible system, Cre recombinase fused to the mutated estrogen receptor is expressed under the control of the CMV early enhancer/chicken beta actin (CAG) promoter (CAG-ER^{T2}CreER^{T2}), in which Cre is only active in the presence of 4-OHT. The inducible GFP overexpression plasmid contains a stop codon flanked by 2 LoxP sites downstream from the CAG promoter, thus GFP is expressed only in the presence of Cre recombinase (CALNL-GFP). CAG-ER^{T2}CreER^{T2} and CALNL-GFP were co-electroporated into the mPFC at embryonic day 15 (E15), and pups were given 4-OHT (2 mg per 100 g body weight) by intraperitoneal injection at P2 to induce GFP expression only after prenatal periods. We found that total numbers of apical and basal dendrites, as well as the number of branches of both apical and basal dendrites were significantly decreased in GFP-labeled pyramidal neurons of NDEL1 CKO mice at P7 (Fig. 2*D*,*E*), which are phenocopied by the genetic deletion of Nos1 (Fig. 1*A*,*B*).

The S-Nitrosylation signaling cascade is regulated by NMDA receptor-mediated neuronal activity-dependent mechanisms which play a major role in regulation of dendritic arborization (Garthwaite et al. 1988; Konur and Ghosh 2005; Nakamura et al. 2013). Nonetheless, the effects of activity-dependent S-Nitrosylation mechanisms for dendritic development have yet to be directly elucidated. We therefore assessed whether neuronal activity modulates S-Nitrosylation of NDEL1 during brain development by using electroconvulsive stimulation (ECS) and observed increased S-Nitrosylation of NDEL1 in the frontal

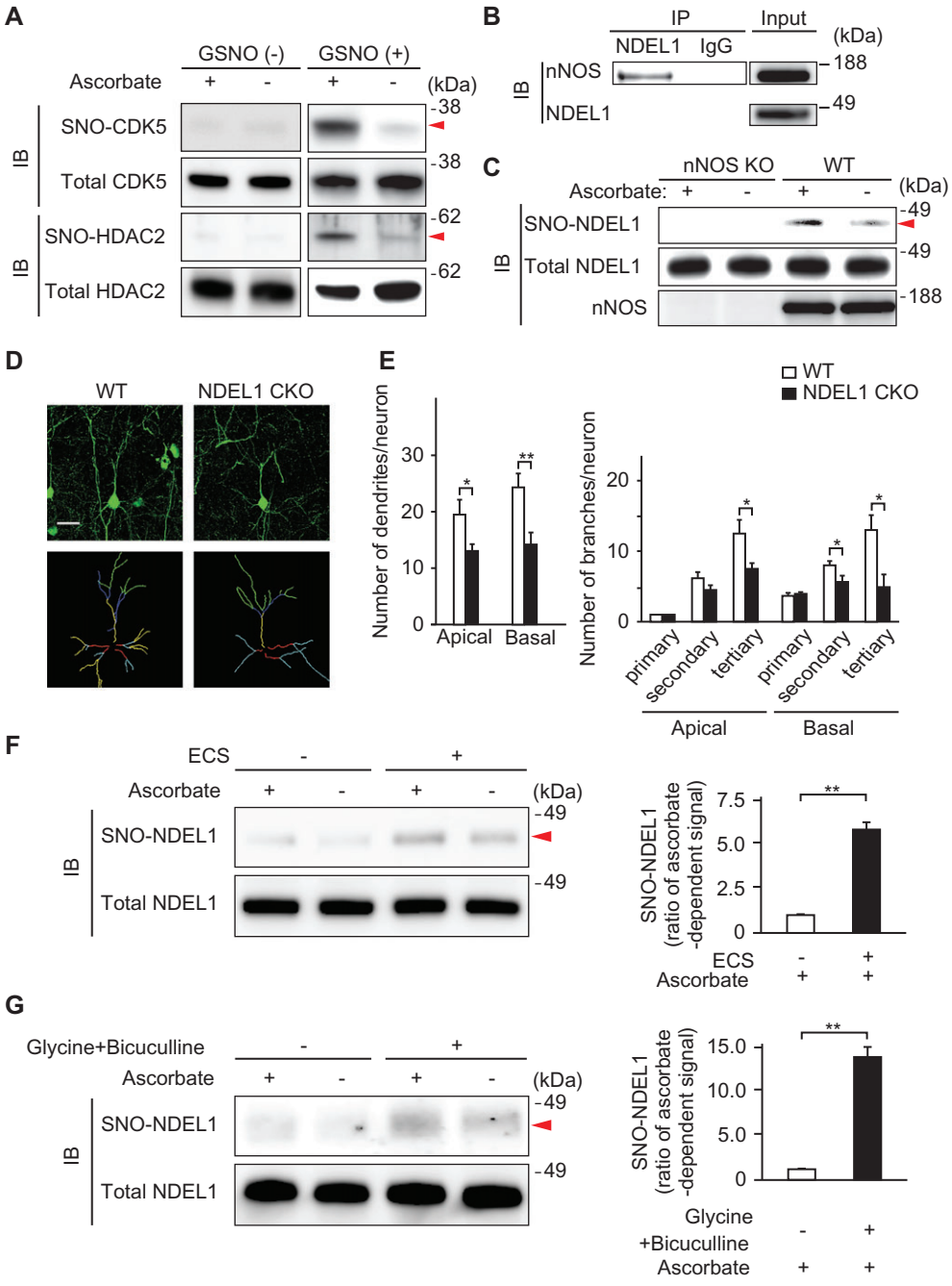

Figure 2. Genetic deletion of Ndel1 impairs dendritic development, and NDEL1 is S-nitrosylated in the developing frontal cortex. (*A*) Protein lysate obtained from the frontal cortex of mice at P7 was analyzed by the biotin-switch assay. Ascorbate promotes biotinylation of S-nitrosothiol, and S-nitrosylated proteins were detected as proteins with biotinylated cysteine. No changes in S-Nitrosylation of CDK5 and HDAC2 were observed although all these proteins were S-nitrosylated by GSNO, an NO donor (arrowhead). IB, immunoblotting. (*B*) Protein interaction of endogenous nNOS with NDEL1 was assessed by co-immunoprecipitation with use of developing primary cortical neurons at 7 DIV. Interaction between nNOS and NDEL1 was observed. Input of each protein is shown. IB indicates an antibody used for western blotting. (*C*) Protein lysates from frontal cortex of homozygous nNOS KO mice and wildtype (WT) littermates at P7 were analyzed by the biotin-switch assay. NDEL1 is S-nitrosylated (SNO^-) in WT mice, but not in nNOS KO mice (arrowhead). (*D* and *E*) Dendrites of GFP-labeled pyramidal neurons in the medial PFC were analyzed in the heterozygous Ndel1 conditional knockout mice (NDEL1 CKO) at P7. NDEL1 CKO mice were electroporated with inducible Cre and GFP expression plasmids (CAG-$ER^{T2}CreER^{T2}$ and CALNL-GFP, respectively) at embryonic day 15 (E15), followed by intraperitoneal injection of 4-OHT at P2. Reduction in the total numbers of apical and basal dendrites ($P = 0.0493$ and $P = 0.002$, respectively), as well as the number of secondary basal branches ($P = 0.0458$) and tertiary branches, both apical and basal ($P = 0.0318$ and $P = 0.0199$, respectively), was observed. Scale bars, 25 μm. Traced dendrites were categorized as primary apical and basal, secondary apical, and basal dendrites, as well as tertiary apical and basal dendrites, respectively. Only neurons with typical morphological characteristics of classic pyramidal neurons (single thick axon arising from the apical side of the soma with multiple basal dendrites and extending vertically to layer I) were analyzed. (*F*) Protein lysate obtained from the frontal cortex of mice at P7 was analyzed by the biotin-switch assay 30 s after electroconvulsive stimulation (ECS) ($n = 3$). Neuronal activity induced by ECS enhances S-Nitrosylation of NDEL1 (arrowhead). (*G*) Primary cortical neurons treated with glycine (200 μM) and bicuculline (20 μM) were assessed by the biotin-switch assay ($n = 3$). Activation of NMDA receptors enhances S-Nitrosylation of NDEL1 (arrowhead). (*E*, *F*, and *G*) $^*P < 0.05$, $^{**}P < 0.01$ determined by Student's *t*-test. Data are presented as the mean ± SEM.

cortex after ECS at P7 (Fig. 2F). To confirm that increased S-Nitrosylation of NDEL1 was caused specifically by NMDA receptor-mediated neuronal activation, we monitored S-Nitrosylation of NDEL1 after stimulation of NMDA receptors with glycine in the developing cortical neurons. Neurons were co-treated with bicuculline, a $GABA_A$ receptor antagonist, as GABA action may regulate dendritic growth during development (Ben-Ari 2002). Activation of NMDA receptors increased S-Nitrosylation of NDEL1 under these conditions (Fig. 2G). These lines of evidence indicate that NDEL1 is S-nitrosylated in an NMDA receptor-mediated activity-dependent manner in the developing frontal cortex.

S-Nitrosylation of NDEL1 at C203 Is Critical for Dendritic Development

NDEL1 has 3 cysteine residues at amino acids 203 (C203), 273 (C273), and 293 (C293) that are evolutionarily conserved between humans and rodents (Supplementary Fig. 4A). The data from in silico analysis through GPS-SNO, the analytical program for prediction of S-Nitrosylation sites (Xue et al. 2010), suggest that C203 is likely to be an S-Nitrosylation consensus motif (Supplementary Fig. 4B). To determine that the C203 residue is responsible for S-Nitrosylation of NDEL1, we created mutant NDEL1 expression constructs, by replacing cysteine with serine (C203S, C273S, and C293S), substitutions that are commonly used for producing S-Nitrosylation-deficient mutants (Thibeault et al. 2010; Selvakumar et al. 2013). NDEL1-WT or these NDEL1 mutants were overexpressed in HEK293 cells. Cell lysates treated with GSNO were then subjected to the biotin-switch assay. As expected, the replacement of C203 with serine (C203S) strongly reduced the S-Nitrosylation of NDEL1, but was not significant with C273 and C293, indicating that C203 is the major site of S-Nitrosylation of NDEL1 (Fig. 3A). To examine the importance of S-Nitrosylation of NDEL1 at the C203 residue in dendritic development, we used a knockdown approach by introducing 2 specific shRNAs against target sequences of mouse Ndel1 (NDEL1 RNAi and NDEL1 RNAi-2) in which knockdown effects on endogenous Ndel1 have previously been characterized (Kamiya et al. 2006). Because mouse NDEL1 exhibits high amino acid sequence homologies to human NDEL1 (96% identity), whereas target sequences of these shRNA to Ndel1 are not identical between human and mouse, a human NDEL1 homolog was used to rescue dendritic phenotypes resulting from the suppression of Ndel1 in developing cortical neurons. Suppression of NDEL1 expression in the developing cortical neurons impaired dendritic branching, consistent with in vivo effects displayed in conditional genetic deletion of Ndel1 (Fig. 2D,E, Supplementary Fig. 5A,B). Importantly, the reduction in number of dendritic branches was seen with 2 independent shRNAs to Ndel1 in a dose-dependent manner and reversed by co-expression with NDEL1-WT, but not with the S-Nitrosylation-deficient mutant NDEL1 (NDEL1-C203S) (Supplementary Fig. 5A,B). Replacing cysteine with tryptophan has recently been reported to mimic S-Nitrosylation (Palmer et al. 2008; Wang et al. 2009). To obtain further evidence supporting the importance of S-Nitrosylation of NDEL1 at C203 for dendritic development, an S-nitrosomimetic mutant of NDEL1 was made by replacing cysteine 203 with tryptophan (NDEL1-C203W). Of note, deficits in dendritic development caused by suppression of NDEL1 expression were restored by co-expression of the S-nitrosomimetic mutant of NDEL1 (Supplementary Fig. 5A,B).

To evaluate the importance of S-Nitrosylation of NDEL1 at C203 residue for dendritic growth exclusively in post-migratory neurons in vivo, we tested the effect of neonatal suppression of NDEL1 expression on dendritic development in the medial PFC by use of the aforementioned Cre/loxP-mediated plasmid system by in utero electroporation (Matsuda and Cepko 2007; Saito et al. 2016). Previous studies including ours have shown the usefulness of in utero electroporation in addressing molecular components in the developing cerebral cortex, via combinations of inducible knockdown approaches and rescue experiments by co-electroporation with multiple expression constructs (LoTurco et al. 2009; Taniguchi et al. 2012; Saito et al. 2016). We made the conditional shRNA against target sequences of Ndel1 in which the knockdown effects have been confirmed (Supplementary Fig. 6) (Kamiya et al. 2006). We also designed an inducible plasmid expressing human NDEL1-WT, NDEL1-C203S, and NDEL1-C203W. Inducible shRNA to Ndel1 or scrambled control shRNA (Control RNAi), together with CALNL-GFP and CAG-ER^{T2}CreER^{T2}, were electroporated into the PFC, followed by intraperitoneal injection of 4-OHT at P2 as described above. When suppression of NDEL1 expression was initiated in the post-migratory neurons, we observed impaired dendritic development in the PFC (Fig. 3B,C). The reduction in number of dendrites and branches was reversed by inducible co-expression with NDEL1-WT, but not with NDEL1-C203S (Fig. 3B,C). Importantly, co-expression of S-nitrosomimetic NDEL1-C203W strongly reversed dendritic deficits caused by suppression of NDEL1 expression, compared with that of WT NDEL1 (Fig. 3B,C).

S-Nitrosylation of NDEL1 at C203 Is Responsible for Dendritic Deficits Caused by Ethanol Exposure

Previous studies have highlighted impairment of NOS activity induced by prenatal ethanol exposure in developing brains (Kimura et al. 1996; Kimura and Brien 1998). In order to examine the effect of alcohol exposure on nNOS signaling and dendritic development, ethanol was administered to neonatal pups (5.0 g/kg body weight) intraperitoneally with a single injection daily from P4 to P7, the period corresponding to human third trimester (Livy et al. 2003; Mooney and Napper 2005; Hamilton et al. 2010). The average of blood alcohol concentrations in mice was 236.73 ± 14.81 mg/dl. We found suppression of NOS enzymatic activity in mice treated with ethanol, whereas no change was observed in expression of nNOS (Fig. 4A,B). Consistently, S-Nitrosylation of NDEL1 was significantly decreased in the frontal cortex by neonatal ethanol exposure (Fig. 4C).

To test whether ethanol exposure during neonatal periods affects dendritic development, dendritic structures were examined in ethanol- or vehicle-treated mice expressing GFP in pyramidal neurons of the medial PFC by utilizing in utero electroporation. We found significant reduction in the total numbers of apical and basal dendrites, the number of primary branches of basal dendrites, as well as the number of tertiary branches, both apical and basal, in GFP-labeled pyramidal neurons of ethanol-treated mice at P7 (Fig. 4D,E). Disruption of S-Nitrosylation of NDEL1 may be a critical nNOS-mediated mechanism underlying dendritic deficits induced by neonatal ethanol exposure. To test this idea, either NDEL1-WT or NDEL1-C203W were neonatally expressed by in utero electroporation in mice treated with ethanol. We observed that dendritic deficits caused by neonatal ethanol exposure were normalized by expression with NDEL1-C203W, but not with NDEL1-WT

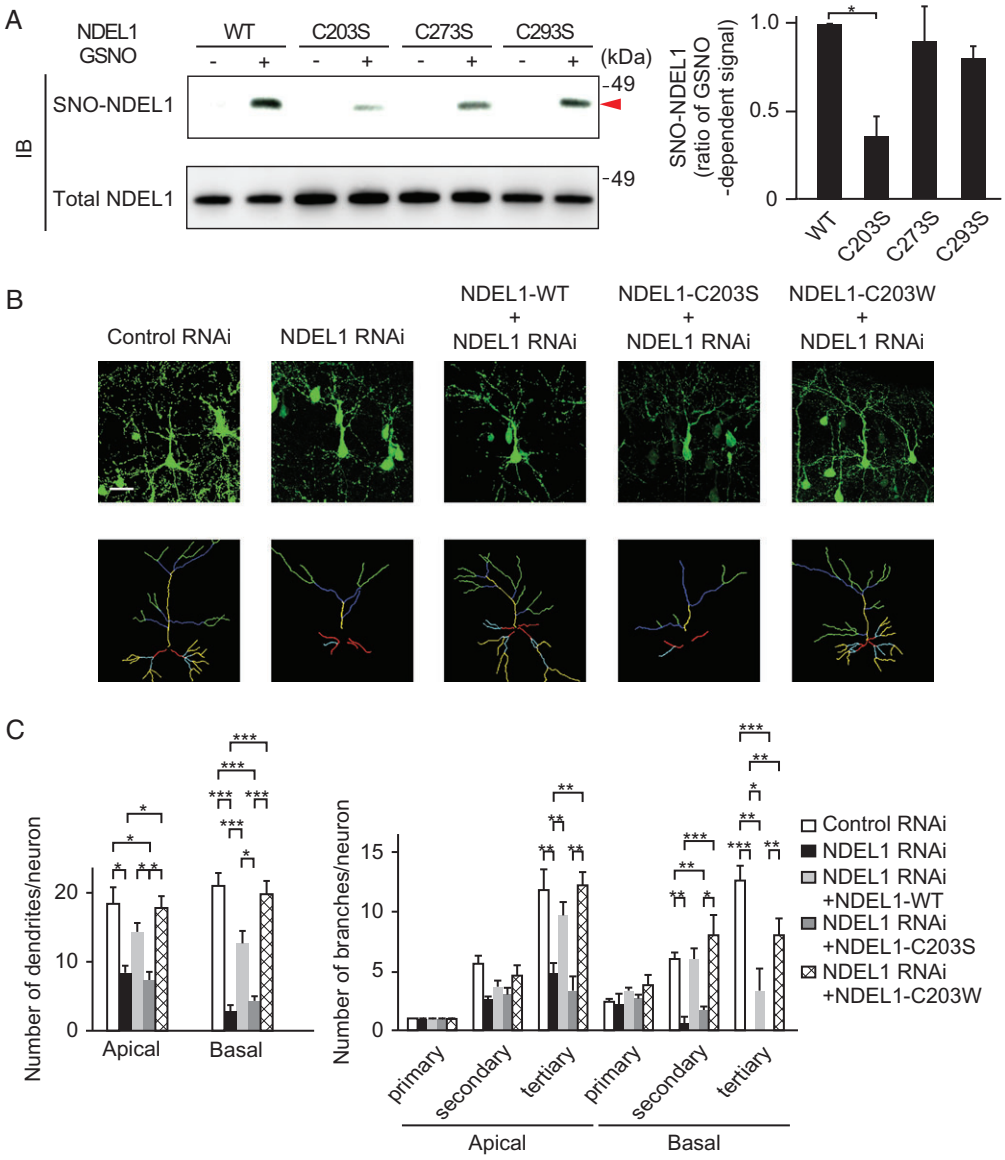

Figure 3. S-Nitrosylation of NDEL1 at C203 is critical for dendritic development. (*A*) HA-tagged WT, as well as mutants with cysteines replaced by serine at amino acid 203 (C203S), 273 (C273S), and 293 (C293S) of NDEL1 were transfected into HEK293 cells. Cell lysates were treated with GSNO, an NO donor (0.2 mM) for 20 min, then subjected to biotin-switch assay ($n = 3$). NDEL1 is S-nitrosylated at cysteine 203, but not at cysteine 273 and 293 (arrowhead). (*B*) Inducible NDEL1 RNAi or control RNAi were co-introduced with CAG-ER^{T2}CreER^{T2} and CALNL-GFP in the PFC followed by intraperitoneal injection of 4-OHT at P2. GFP-labeled pyramidal neurons with neonatal knockdown of NDEL1 in the medial PFC are shown. Traced dendrites were categorized as primary apical and basal, secondary apical, and basal dendrites, as well as tertiary apical and basal dendrites, respectively. Scale bars, 25 μm. Only GFP-labeled neurons with typical morphological characteristics of classic pyramidal neurons (single thick axon arising from the apical side of the soma with multiple basal dendrites and extending vertically to layer I) were analyzed. (*C*) Total numbers of apical and basal dendrites ($F(4,18) = 6.835$, $P = 0.0016$ and $F(4,18) = 21.89$, $P < 0.0001$, respectively), as well as branches of tertiary apical dendrites ($F(4,18) = 8.204$, $P = 0.0006$) and secondary, tertiary basal dendrites ($F(4,18) = 10.9$, $P = 0.0001$ and $F(4,18) = 16.12$, $P < 0.0001$, respectively) are significantly reduced in neonatal NDEL1-suppressed mice in which phenotypes were partially normalized by inducible co-expression with WT NDEL1, but not with the C203S mutant. C203W mutant substantially rescued dendritic deficits caused by suppression of NDEL1. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ determined by one-way ANOVA with post hoc Tukey–Kramer test (*A* and *C*). Data are presented as the mean ± SEM.

(Fig. 4*D*,*E*). These results indicate that specific up-regulation of S-Nitrosylation of NDEL1 during the neonatal period is sufficient to ameliorate dendritic deficits caused by neonatal ethanol exposure.

Discussion

This is the first study, to our knowledge, demonstrating the roles for activity-dependent S-Nitrosylation in neonatal cerebral cortex development. Our data indicate that nNOS contributes to the regulation of dendritic development via S-Nitrosylation-mediated mechanisms, whereas the sGC/cGMP pathway does not appear to be involved in a major way under in vivo conditions. Previous studies implicated the sGC/cGMP pathway in developmental cellular processes in vitro and in invertebrate models (Cramer et al. 1996; Hindley et al. 1997; Gibbs and Truman 1998; Nikonenko et al. 2008; Stern and Bicker 2008; Lee et al. 2009; Shelly et al. 2010). In contrast, multiple inhibitors of the sGC/cGMP pathway did not affect dendritic branching, particularly in the medial PFC during the

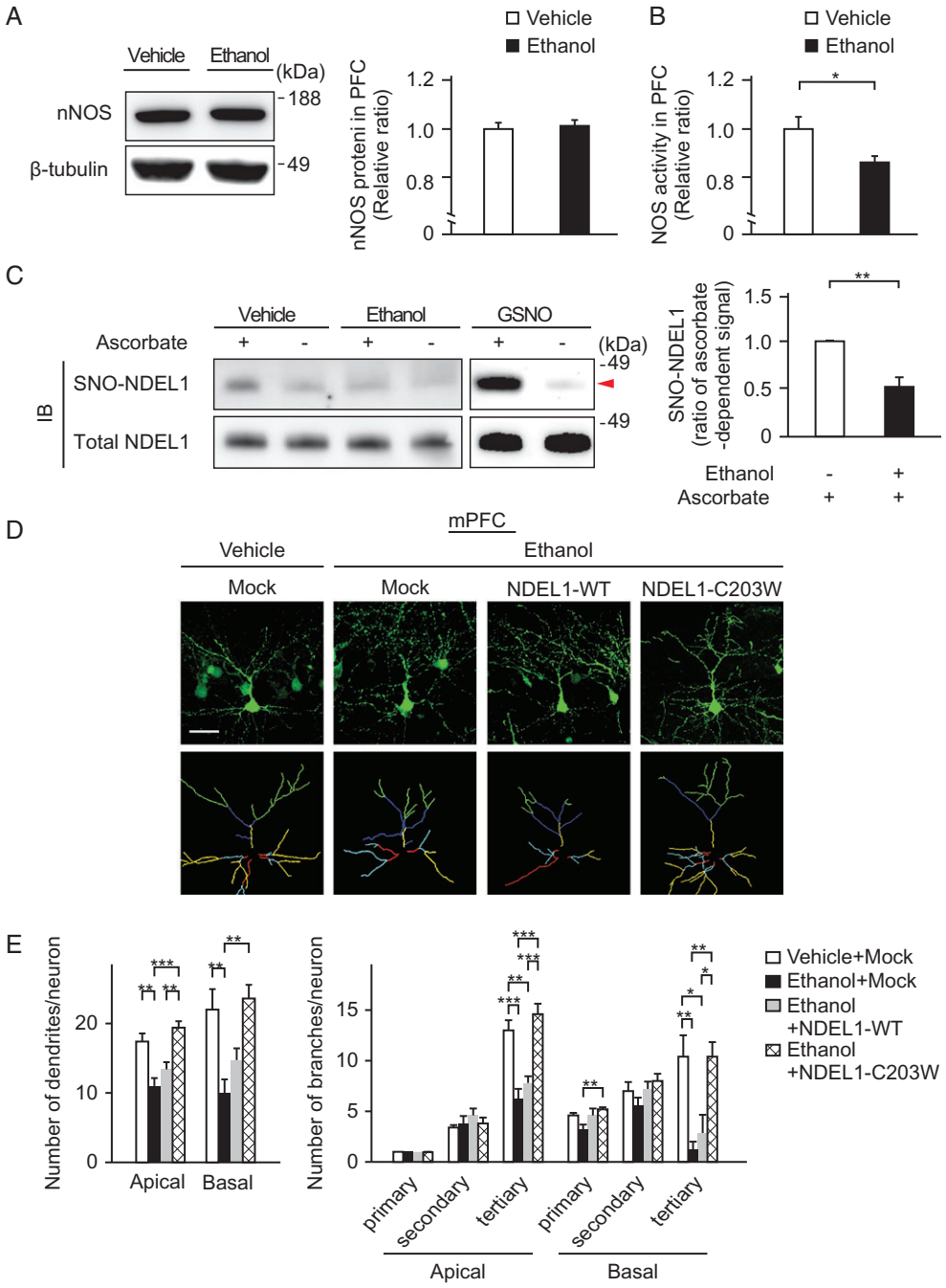

Figure 4. Ethanol exposure during the human third trimester-equivalent period impairs S-Nitrosylation of NDEL1 and dendritic development in the PFC. (*A*) The effect of neonatal ethanol exposure on protein expression of nNOS was assessed. The signal intensity was normalized by the internal control (β-tubulin). No difference was observed in nNOS expression in the PFC between vehicle- and ethanol-administered groups. (*B*) The enzymatic activity of NOS in the PFC was measured by colorimetric assay. The NOS enzymatic activity was significantly decreased by neonatal ethanol exposure ($P = 0.0229$). (*C*) Protein lysate obtained from the frontal cortex of mice at P7 was analyzed by the biotin-switch assay with or without ethanol administration. Ascorbate promotes biotinylation of S-nitrosothiol, and S-nitrosylated proteins were detected as proteins with biotinylated cysteine. S-Nitrosylation of NDEL1 was significantly decreased by ethanol treatment (arrowhead). GSNO, an NO donor. IB, immunoblotting, was used for positive control of S-Nitrosylation of NDEL1. (*D* and *E*) The effect of postnatal expression of NDEL1-WT or NDEL1-C203W on dendritic deficits was characterized in the medial PFC in mice subjected to third trimester-equivalent ethanol administration. Dendrites of GFP-labeled pyramidal neurons in the medial PFC were analyzed at postnatal day P7, when vigorous dendritic growth occurs. Reduction in the total numbers of apical and basal dendrites ($F(3,16) = 18.23$, $P < 0.0001$ and $F(3,16) = 12.61$, $P = 0.0002$, respectively), the number of primary branches of basal dendrites ($F(3,16) = 3.6$, $P = 0.0368$), as well as the number of tertiary branches, both apical and basal ($F(3,16) = 18.38$, $P < 0.0001$ and $F(3,16) = 9.036$, $P = 0.001$, respectively), was observed in the animals treated with ethanol. These reductions were normalized by inducible co-expression with NDEL1-C203W, but not with NDEL1-WT. Scale bars, 25 μm. Traced dendrites were categorized as primary apical and basal, secondary apical, and basal dendrites, as well as tertiary apical and basal dendrites, respectively. Only neurons with typical morphological characteristics of classic pyramidal neurons (single thick axon arising from the apical side of the soma with multiple basal dendrites and extending vertically to layer I) were analyzed. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ determined by Student's *t*-test (*A*, *B*, and *C*) or one-way ANOVA with post hoc Tukey–Kramer test (*E*). Data are presented as the mean ± SEM.

neonatal period. This discrepancy may be explained by the possibility that unknown compensatory mechanisms overcome dendritic deficits caused by inhibition of sGC/cGMP pathway in vivo.

Recent studies have begun to shed light on mechanisms underlying the effect of fetal alcohol exposure on brain development (Sulik 2014). Transcriptome analysis revealed that alcohol exposure during prenatal periods equivalent to second trimester of human pregnancy (Mooney et al. 2004; Schneider et al. 2011; Bake et al. 2012) alters expression of multiple molecules such as SATB2, ZEB2, heat shock proteins, and Heat Shock Factor1 which are involved in neuronal development and survival (Hashimoto-Torii et al. 2011). In addition, the detrimental effect of alcohol exposure during early postnatal periods in rodents, which is equivalent to the human third trimester periods, has been recently investigated (Ikonomidou et al. 2000; Granato et al. 2003; Livy et al. 2003; Mooney and Napper 2005; Hamilton et al. 2010; Diaz et al. 2014). Note that in this model, neonatal pups are directly exposed to ethanol, not by a placental-fetal unit which is the route of alcohol exposure in humans. Nonetheless, exploring the effect of neonatal alcohol exposure in rodents is important to decipher molecular mechanisms underlying the effect of alcohol on brain development in the period equivalent to human third trimester (Wilson and Cudd 2011). For instance, Bonthius et al. reported that neonatal ethanol exposure induced neuronal loss during cerebral cortex development (Bonthius et al. 2002; de Licona et al. 2009). The same group also demonstrated that neuronal loss induced by ethanol exposure was worsened by genetic deletion of Nos1, which was rescued by activation of sGC/cGMP pathway in cerebellar granule neuron cultures (Bonthius et al. 2003, 2006, 2008, 2009; Karacay et al. 2007). Nonetheless, our results demonstrate that pharmacological blockade of sGC/cGMP pathway has no detrimental effect on dendritic development in the developing medial PFC.

In order to identify S-nitrosylated proteins during neonatal periods, we applied a candidate target approach; in particular, we focused on molecules which had reportedly been S-Nitrosylated during early developmental periods, because unbiased screening approaches in protein samples from brains are not technically feasible for identification of endogenously S-nitrosylated proteins. Although S-Nitrosylation of CDK5 and HDAC2 are required for embryonic brain development (Nott et al. 2008, 2013; Zhang et al. 2010), we found that this is not the case for neonatal cortical development. Instead, our data demonstrate that S-Nitrosylation of NDEL1 specifically at cysteine 203 is one such mechanism to regulate activity-dependent dendritic development during the neonatal period. Importantly, dendritic abnormalities caused by neonatal ethanol exposure were normalized by up-regulation of S-Nitrosylation of NDEL1, suggesting that disruption of S-Nitrosylation at C203 of NDEL1 may act as a pathological mechanism underlying dendritic deficits caused by alcohol exposure. While much evidence suggests that NDEL1 participates in diverse cellular functions, including cytoskeletal organization and intracellular transport (Chansard et al. 2011), molecular mechanisms underlying the downstream actions of S-Nitrosylation of NDEL1 for regulation of dendritic development are still unclear. It also remains an open question whether dendritic deficits induced by disruption of S-Nitrosylation of NDEL1 could have long lasting effects on adult behaviors. Recent studies reported that NOS1AP/CAPON, a binding protein of nNOS, is involved in dendritic development (Carrel et al. 2009, 2015; Richier et al. 2010; Candemir et al. 2016; Hernandez et al. 2016). Thus, it would also be of interest to investigate how activity-dependent S-nitrosylated modifications of other molecules are involved in deficits in dendritic development caused by alcohol exposure. These lines of study may ultimately provide the foundation for developing early interventions for individuals at risk in FASDs.

NOS1 and genes in nNOS signaling, such as NOS1AP, are genetically associated with multiple psychiatric disorders, such as schizophrenia, attention-deficit/hyperactivity disorder, and major depressive disorder (O'Donovan et al. 2008; Freudenberg et al. 2015; Kudlow et al. 2016). The importance of nNOS for brain function, including cognition has also been reported in human and preclinical studies (Nelson et al. 1995; Reif et al. 2006, 2009; Zoubovsky et al. 2011). Thus, altered nNOS signaling may contribute to pathophysiologies not only of FASD but also of other psychiatric conditions.

Authors' Contributions

At.S. performed all experiments. Y.T. performed immunocytochemistry, immunohistochemistry, and dendrite analysis. S-H.K. and B.S. performed and assisted with biotin-switch assay. M.D.B., G.P., and S.R. performed in utero electroporation. G.P. performed immunohistochemistry and dendrite analysis. M.J. performed western blotting. J.S., P.Y., and X.Z. assisted in experiments. S.H. and A.W.-B. provided NDEL1 CKO mice and assisted with data interpretation. S.H.S. and Ak.S. assisted with research design and data interpretation. A.K. designed and directed the project and wrote the manuscript.

Supplementary Material

Supplementary material can be found at: http://www.cercor.oxfordjournals.org/.

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