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Promoter methylation represses AT₂R gene and increases brain hypoxic-ischemic injury in neonatal rats

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Abstract

Perinatal nicotine exposure downregulated angiotensin II type 2 receptor (AT₂R) in the developing brain and increased brain vulnerability to hypoxic-ischemic injury in male neonatal rats. We tested the hypothesis that site-specific CpG methylation at AT₂R gene promoter contributes to the increased vulnerability of brain injury in the neonate. Nicotine was administered to pregnant rats from day 4 of gestation to day 10 after birth. Brain hypoxic-ischemic injury was induced in day 10 male pups. CpG methylation at AT₂R promoter was determined in the brain by quantitative methylation-specific PCR. Nicotine exposure significantly increased methylation of a single CpG₋₅₂ locus near the TATA-box at AT₂R promoter. Electrophoretic mobility shift assay indicated that methylation of CpG₋₅₂ significantly decreased the binding affinity of TATA-binding protein (TBP). Chromatin immunoprecipitation assay further demonstrated an increase in the binding of a methyl-binding protein and a decrease in TBP binding to AT₂R promoter *in vivo* in neonatal brains of nicotine-treated animals. This resulted in AT₂R gene repression in the brain. Intracerebroventricular administration of a demethylating agent 5-aza-2'-deoxycytidine abrogated the enhanced methylation of CpG₋₅₂, rescued the TBP binding, and restored AT₂R gene expression. Of importance, 5-aza-2'-deoxycytidine reversed the nicotine-increased vulnerability of brain hypoxic-ischemic injury in the neonate. The finding provides mechanistic evidence of increased promoter methylation and resultant AT₂R gene repression in the developing brain linking perinatal stress and a pathophysiological consequence of heightened vulnerability of brain hypoxic-ischemic encephalopathy in the neonate.

Keywords

nicotine; AT₂R; methylation; hypoxic-ischemic encephalopathy

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Disclosures

None.

Introduction

Hypoxic-ischemic encephalopathy (HIE) is the most common cause of newborn brain damage due to systemic asphyxia, which may occur prior, during or after birth. HIE causes severe mortality and long-lasting morbidity including cerebral palsy, seizure, and cognitive retardation in infants and children (Ferrieo, 2004; Verklan, 2009). Emerging evidence suggests that aberrant brain development due to fetal stress may underpin the pathogenesis of HIE (Jensen, 2006). Maternal smoking is the single most widespread perinatal insult in the world and it has been associated with adverse pregnancy outcomes for mother, fetus and the newborn. Recent studies have provided evidence linking perinatal nicotine exposure and the increased incidence of neurodevelopmental disorders, neurobehavioral deficits, impaired cognitive performance, and increased risk of affective disorders later in life (Wickstrom, 2007; Pauly and Slotkin, 2008). Indeed, our recent study in a rat model has demonstrated that perinatal nicotine exposure suppresses angiotensin II type 2 receptor (AT₂R) expression in the developing brain, resulting in an increase in the vulnerability of HIE brain injury in a sex-dependent manner in male neonates (Li et al., 2012).

The mechanisms underlying perinatal nicotine-mediated AT₂R gene repression in the developing brain remain elusive. Recent studies suggested that CpG methylation in non-CpG island, sequence-specific transcription factor binding sites played an important role in epigenetic modification of gene expression patterns in the developing fetus in response to perinatal stress (Lawrence et al., 2011; Meyer et al., 2009; Patterson et al., 2010; Xiong et al., 2010). DNA methylation is a chief mechanism for epigenetic modification of gene expression patterns and occurs at cytosine in the CpG dinucleotide sequence (Jaenisch and Bird, 2003; Jones and Takai, 2001; Reik and Dean, 2001). Methylation in promoter regions is generally associated with the repression of transcription, leading to a long-term shutdown of the associated genes. Methylation of CpG islands in gene promoter regions alters chromatin structure and transcription. Similarly, methylation of a single CpG dinucleotide at sequence-specific transcription factor binding sites may repress gene expression through changes in the binding affinity of transcription factors by altering the major groove structure of DNA to which the DNA binding proteins bind (Campanero et al., 2000; Fujimoto et al., 2005; Zhu et al., 2003), as well as by recruiting methyl-CpG binding proteins (MBPs) (Jones and Laird, 1999; Wade, 2001). Rat AT₂R gene promoter has a TATA element at -48 from the transcription start site, and a single CpG₋₅₂ locus 3 bases upstream of the TATA-box is identified at the AT₂R promoter (Xue et al., 2011). It has been suggested that increased methylation of a single CpG locus 3 bases upstream of TATA-box represses gene expression (Kitazawa and Kitazawa, 2007). Herein, we present evidence that perinatal nicotine exposure increases methylation of a single CpG₋₅₂ locus near the TATA element at AT₂R gene promoter, resulting in a decrease in the binding of TATA-binding protein (TBP) to AT₂R promoter and a repression of AT₂R gene expression in the developing brain. Of importance, a demethylating agent 5-aza-2'-deoxycytidine abrogated nicotine-induced CpG₋₅₂ methylation, rescued the TBP binding, restored AT₂R expression, and reversed the heightened vulnerability of HIE in neonatal brains.

Materials and Methods

Experimental animals

Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and were randomly divided into 2 groups: 1) saline control; and 2) nicotine administration through osmotic minipumps (4 µg/kg/min) implanted subcutaneously from Day 4 of gestation to Day 10 after birth, as previously described (Xiao et al., 2007). Briefly, on the 4th day of pregnancy, rats were anesthetized with 2% isoflurane. An incision was made on the back to insert osmotic minipumps (type 2ML4, Alza Corp). The incision was

closed with four sutures. Half of pregnant rats were implanted with minipumps containing nicotine and the other half with minipumps containing only saline serving as the vehicle control. The infusion lasted for 28 days to the pregnant rats and to the lactating mother until day 10 after delivery. Rats were allowed to give birth and studies were conducted in 10-day-old (P10) male pups. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Brain hypoxic-ischemic (HI) treatment and intracerebroventricular injection

Pups received intracerebroventricular injection of 5-aza-2'-deoxycytidine (1 mg/kg; Sigma-Aldrich) or saline control at day 7, as previously described (Li et al., 2012). Briefly, pups were anesthetized with 2% isoflurane and fixed on a stereotaxic apparatus (Stoelting, Wood Dale, IL). An incision was made on the skull surface and bregma was exposed. 5-Aza-2'-deoxycytidine was injected at a rate of 1 μ L/minutes with a 10 μ L syringe (Stoelting) on the right hemisphere following the coordinates relative to bregma: 2.0 mm posterior, 1.5 mm lateral, and 3.0 mm below the skull surface (Han and Holtzman, 2000). The injection lasted 2 minutes and the needle was kept for additional 5 minutes before its removal. The incision was sutured. Brain HI treatment with a modified Rice-Vannucci model was performed at day 10, as previously reported (Li et al., 2012). Briefly, pups were anesthetized and the right common carotid artery was ligated. After recovery for 1 hour, pups were treated with 8% O₂ for 1.5 hours.

Measurement of infarct size

Pups were euthanized 48 hours after the HI treatment. Coronal slices of the brain (2-mm thick) were cut and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride monohydrate for 5 minutes at 37°C, followed by fixation with 10% formaldehyde overnight. Each slice was weighed and photographed separately. The infarction area was analyzed by Image J software (Version 1.40; National Institutes of Health, Bethesda, MD), corrected by the slice weight, summed for each brain, and expressed as a percentage of whole brain weight.

Western immunoblotting

Brains were homogenized in a lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris HCl, 10 mmol/L EDTA, 0.1% Tween-20, 1% Triton, 0.1% β -mercaptoethanol, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 μ g/mL leupeptin, and 5 μ g/mL aprotinin, pH 7.4. Homogenates were centrifuged at 4°C for 10 minutes at 10,000 g, and supernatants collected. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). Samples with equal amounts of protein were loaded onto 10% polyacrylamide gel with 0.1% sodium dodecyl sulfate and separated by electrophoresis at 100 V for 120 minutes. Proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies against AT₂R (1:1000; Santa Cruz Biotechnology; Santa Cruz, CA) as described previously (Li et al., 2012). After washing, membranes were incubated with secondary horseradish peroxidase conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with Kodak ID image analysis software. Band intensities were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real-time RT-PCR

RNA was extracted from brains and abundance of AT₂R mRNA was determined by real-time RT-PCR using an Icyler Thermal cycler (Bio-Rad, Hercules, CA), as described previously (Li et al., 2012). The AT₂R primers used were: 5'-caatctggctgtgctgactt-3

(forward) and 5'-tgcatcatcacaggtccaaaga-3' (reverse). Real-time RT-PCR was performed in a final volume of 25 μ L. Each polymerase chain reaction mixture consisted of 600 nmol/L of primers, 33 U of M-MLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad) containing 0.625 U Taq polymerase, 400 μ mol/L each of dATP, dCTP, dGTP, and dTTP, 100 mmol/L KCl, 16.6 mmol/L ammonium sulfate, 40 mmol/L Tris-HCl, 6 mmol/L MgSO₄, SYBR Green I, 20 nmol/L fluorescing, and stabilizers. The following reverse transcription-polymerase chain reaction protocol was used: 42°C for 30 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 20 seconds, 56°C for 1 minute, 72°C for 20 seconds. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference and serial dilutions of the positive control was performed on each plate to create a standard curve. Polymerase chain reaction was performed in triplicate, and threshold cycle numbers were averaged.

Quantitative methylation-specific PCR (MSP)

Genomic DNA was isolated from brains using a GenElute Mammalian Genomic DNA Mini-Prep kit (Sigma), denatured with 2 N NaOH at 42°C for 15 minutes, treated with sodium bisulfite at 55°C for 16 hours, purified by a Wizard DNA clean up system (Promega), and re-suspended in 40 μ L of H₂O. Bisulfite-treated DNA was used as a template for real-time fluorogenic methylation-specific PCR at the CpG₋₅₂ near the TATA-box at AT₂R promoter (forward primer, 5'-tttttggaagtgtgtaagtgtta-3'; reverse primer for C, 5'-ctctaattctcttcttatattca-3'; reverse primer for ^mC, 5'-ctctaattctcttcttatattcg-3'), as described previously (Li et al., 2012). Real-time MSP was performed using the iQ SYBR Green Supermix with iCycler (Bio-Rad). Data are presented as the percent of methylation at the region of interest (methylated CpG/methylated CpG + unmethylated CpG $\times$ 100), as described previously (Lawrence et al., 2011; Patterson et al., 2010).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from brains using NXTRACT CellLytic Nuclear Extraction Kit (Sigma). The oligonucleotide probes with CpG₋₅₂ and ^mCpG₋₅₂ of the TBP binding site at AT₂R promoter were labeled and subjected to gel shift assay using the Biotin 3' end labeling kit and Light-Shift Chemiluminescent EMSA kit (Pierce Biotechnology, Rockford, IL), as previously described (Meyer et al., 2009; Patterson et al., 2010). Briefly, single stranded oligos were incubated with Terminal Deoxynucleotidyl Transferase (TdT) and Biotin-11-dUTP in binding mixture for 30 minutes at 37°C. The TdT adds a biotin labeled dUTP to the 3'-end of the oligonucleotides. The oligos were extracted using chloroform and isoamyl alcohol to remove the enzyme and unincorporated biotin-11-dUTP. Dot blots were performed to ensure the oligos were labeled equally. Combining sense and antisense oligos and exposing to 95°C for 5 min was done to anneal complementary oligos. The labeled oligonucleotides were then incubated with or without nuclear extracts in the binding buffer (from Light-Shift kit). Binding reactions were performed in 20 μ L containing 50 fmol oligonucleotide probes, 1 $\times$ binding buffer, 1 μ g of poly (dI-dC), and 10 μ g of nuclear extracts. For competitions studies, increasing concentrations of non-labeled oligonucleotides were added to binding reactions. For super-shift assay, 2 μ L of affinity purified TBP antibody (Active Motif) was added to the binding reaction. The samples were then run on a native 5% polyacrylamide gel. The contents of the gel were then transferred to a nylon membrane (Pierce) and crosslinked to the membrane using a UV crosslinker (125 mJoules/cm²). Membranes were blocked and then visualized using the reagents provided in the LightShift kit.

Chromatin immunoprecipitation assay (ChIP)

Chromatin extracts were prepared from pup brains. ChIP assays were performed using the ChIP-IT kit (Active Motif), as previously described (Meyer et al., 2009; Patterson et al.,

2010). Briefly, brain tissues were incubated with 1% formaldehyde for 10 min to crosslink and maintain DNA/protein interactions. After the reactions were stopped with glycine, tissues were washed, and chromatin was isolated and sheared into medium fragments (200–1000 base pairs) using a sonicator. ChIP reactions were performed using an antibody against TBP or MeCP2 to precipitate the transcription factor/DNA complex. Crosslinking was then reversed using a salt solution and the proteins were digested with proteinase K. Primer flanking the TBP binding site were used for quantitative RT-PCR: 5'-tctgaaagctggcaagtgt-3' (forward) and 5'-tgggatgtaactgcaccaga-3' (reverse). PCR amplification products were visualized on 3% agarose gel stained with ethidium bromide. To quantify PCR amplification, 45 cycles of real-time PCR were carried out with 3 min initial denaturation followed by 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad, Hercules, CA). All reactions were repeated in triplicate and the results were calculated as the ratio of immunoprecipitated DNA over input DNA.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Experimental number (n) represents neonates from different dams. Statistical significance ($P < 0.05$) was determined by analysis of variance followed by Neuman-Keuls post hoc testing or Student t test, where appropriate.

Results

Methylation of CpG₋₅₂ locus inhibited TBP binding affinity

Previously, we demonstrated that deletion of the TATA-box at rat AT₂R promoter region resulted in a significant decrease in AT₂R promoter activity (Xue et al., 2011). To demonstrate the binding of TATA-binding protein (TBP) to the TATA element at AT₂R promoter, electrophoretic mobility shift assays were performed. Incubation of nuclear extracts from pup brains with double-stranded oligonucleotide probes encompassing the TATA element resulted in the appearance of a major DNA-protein complex (Figure 1, lane 2), which was blocked by 200-fold excess of unlabeled oligonucleotide probes in cold competition (Figure 1, lane 4). Super-shift analysis showed that a TBP antibody caused super-shifting of the DNA-protein complex (Figure 1, lane 3). A single CpG₋₅₂ locus 3 bases upstream of the TATA-box was identified at rat AT₂R promoter (Xue et al., 2011). To determine whether methylation of CpG₋₅₂ locus inhibits TBP binding, the binding affinity of TBP to oligonucleotide probes with the TATA element containing either methylated or unmethylated CpG₋₅₂ locus was determined by competitive EMSA performed in pooled nuclear extracts from pup brains with the increasing ratio of unlabeled/labeled oligonucleotides encompassing the TATA element. As shown in Figure 2, methylation of CpG₋₅₂ locus resulted in a significant decrease in the TBP binding affinity to the TATA element.

5-Aza-2'-deoxycytidine abrogated nicotine-induced methylation of CpG₋₅₂ locus and restored AT₂R expression

The previous study demonstrated that perinatal nicotine exposure resulted in a down-regulation of AT₂R expression in the developing brain (Li et al., 2012). To determine the causal role of CpG₋₅₂ locus methylation in the nicotine-mediated down-regulation of AT₂R in pup brains, we measured methylation status of the CpG₋₅₂ locus at AT₂R promoter in male pups in the control and nicotine-treated animals. As shown in Figure 3, the nicotine treatment significantly increased methylation of the CpG₋₅₂ locus. Of importance, the treatment of pups with a DNA demethylating agent 5-aza-2'-deoxycytidine abrogated the nicotine-induced methylation (Figure 3). We further investigated the functional significance of the nicotine-mediated methylation in regulating TBP binding to AT₂R promoter *in vivo*

in the context of intact chromatin *via* a ChIP approach. As shown in Figure 4, the increased methylation of CpG₋₅₂ locus by nicotine resulted in a significant increase in the binding of MeCP2 and a decrease in the binding of TBP to the TATA element at AT₂R promoter in pup brains. 5-aza-2'-deoxycytidine blocked these nicotine-induced effects (Figure 4). Consistently, 5-aza-2'-deoxycytidine restored the nicotine-induced down-regulation of AT₂R mRNA and protein expression in the brains (Figure 5).

5-Aza-2'-deoxycytidine rescued nicotine-induced vulnerability of HI injury in pup brains

AT₂R played a critical role in protecting neonatal brains from HI injury (Li et al., 2012). We thus investigated the causal role of nicotine-induced epigenetic down-regulation of AT₂R in the heightened vulnerability of pup brains to HI injury by determining whether 5-aza-2'-deoxycytidine-mediated restoration of AT₂R expression in the developing brain reversed nicotine-induced vulnerability of HI injury in pup brains. As shown in Figure 6, in the absence of 5-aza-2'-deoxycytidine, the nicotine treatment resulted in a significant increase in HI injury in pup brains, which was abolished by 5-aza-2'-deoxycytidine. Whereas the nicotine treatment decreased the body weight (15.9 ± 1.4 g *vs.* 18.4 ± 0.4 g, $P < 0.05$) but increased the brain to body weight ratio (0.06 ± 0.00 *vs.* 0.05 ± 0.00 , $P < 0.05$) in the pups, the treatment of 5-aza-2'-deoxycytidine had no significant effect on the gross development of neonates in either control or nicotine-treated groups. Thus, the body weight in the absence or presence of 5-aza-2'-deoxycytidine were 18.4 ± 0.4 g *vs.* 17.8 ± 0.4 g ($P > 0.05$) in control pups, and 15.9 ± 1.4 g *vs.* 15.7 ± 1.0 g ($P > 0.05$) in nicotine-treated animals. The brain to body weight ratio in the absence or presence of 5-aza-2'-deoxycytidine were 0.05 ± 0.00 *vs.* 0.05 ± 0.00 ($P > 0.05$) in control pups, and 0.06 ± 0.00 *vs.* 0.06 ± 0.00 ($P > 0.05$) in nicotine-treated animals.

Discussion

The present study reveals evidence that heightened methylation of a single CpG₋₅₂ locus adjacent to the TATA element at AT₂R promoter significantly inhibits the binding activity of TBP and suppresses AT₂R mRNA and protein expression in the developing brain in response to perinatal nicotine exposure. Of importance, the findings that DNA demethylating agent 5-aza-2'-deoxycytidine blocked nicotine-induced methylation, restored AT₂R expression, and rescued the heightened brain susceptibility to HI injury in pups, provide novel evidence of a causal role of gene-specific promoter methylation in perinatal stress-mediated HIE vulnerability in the neonate.

In the previous study, we have reported in a rat model that maternal nicotine administration increases HIE-induced brain injury in male but not female rat pups *via* reprogramming the expression patterns of AT₂R in a sex-specific manner in the developing brain (Li et al., 2012). Immunofluorescence and confocal imaging analyses showed that AT₂R mainly presented in neurons, but not in astrocytes, of the cortex and hippocampus in P10 pups (Li et al., 2012). The neuroprotective effect of AT₂R was thought mainly through its neuronal action (Laflamme et al., 1996; Mogi et al., 2006; McCarthy et al., 2009). Nicotine treatment significantly repressed expression levels of AT₂R mRNA and protein in the brain of male pups but up-regulated its expression in female pups, demonstrating a sex-specific effect. The finding that AT₂R agonist CGP42112 reversed the nicotine-induced increase in brain HI injury demonstrated an important role of brain AT₂R repression in programming of enhanced vulnerability of neonatal HIE. However, the underlying molecular mechanisms of perinatal nicotine exposure in repressing AT₂R gene transcription in neonatal brains remained elusive.

Rat AT₂R gene promoter has a TATA element at -48 from the transcription start site (Xue et al., 2011). In the present study, we demonstrated that an antiserum to TATA-box binding

protein caused super-shifting of the DNA-protein complex resulting from the binding of nuclear extracts from pup brains with the double-stranded oligonucleotide probes containing the TATA element, indicating a consensus TATA binding site at AT₂R promoter in rat brains. The functional significance of the TATA element in the regulation of rat AT₂R gene activity was demonstrated by the finding that deletion of TATA significantly decreased the AT₂R promoter activity (Xue et al., 2011). The present finding that methylation of a single CpG₋₅₂ locus 3-base upstream of the TATA-box significantly decreased the binding affinity of TATA-box binding protein to the TATA element is intriguing and indicates an important epigenetic mechanism of CpG methylation at a sequence-specific binding site in inhibiting transcription factor binding and a gene repression in the developing brain. Although the transcriptional regulation by DNA methylation is often observed in CpG islands located around the promoter region *via* the sequence-nonspecific and methylation-specific binding of inhibiting methylated CpG-binding proteins (Jones and Laird, 1999; Wade, 2001), DNA methylation of sequence-specific transcription factor binding sites can alter gene expression through changes in the binding affinity of transcription factors by altering the major groove structure of DNA to which the DNA-binding proteins bind (Campanero et al., 2000; Fujimoto et al., 2005; Zhu et al., 2003). In agreement with the present finding, previous studies demonstrated that fetal stress resulted in an increase in sequence-specific CpG methylation at Sp1 and Egr1 binding sites at protein kinase C gene (PKC) promoter and PKC gene repression in the developing heart (Lawrence et al., 2011; Meyer et al., 2009; Patterson et al., 2010). In addition, it has been demonstrated that increased methylation at a CpG locus 3 bases upstream of TATA-box inhibits the binding of the TATA-box binding protein and decreases receptor activator of nuclear factor- κ B ligand gene promoter activity (Kitazawa and Kitazawa, 2007).

The finding that nicotine treatment significantly increased methylation of CpG₋₅₂ locus 3 bases upstream of TATA-box at the AT₂R promoter in male pup brains reveals an important mechanism of site-specific CpG methylation in epigenetic repression of AT₂R gene in the developing brain in a sex-dependent manner. This notion is further supported by the results of chromatin immunoprecipitation assays in the present study, demonstrating that the nicotine-induced increase in methylation of the CpG₋₅₂ locus inhibited the binding of TATA-box binding protein to the TATA element at the AT₂R promoter *in vivo* in pup brains in the context of intact chromatin. As a control, our previous study demonstrated that the nicotine treatment had no significant effect on CpG₋₅₂ locus methylation at the AT₂R promoter in female pup brains (Li et al., 2012). A mechanism of CpG methylation-mediated inhibition of transcription factor binding is *via* the binding of methyl-CpG binding proteins (MBPs) (Jones and Laird, 1999; Wade, 2001). MBPs that bind to single or multiple CpGs interact with a co-repressor complex containing histone deacetylases and other chromatin remodeling factors, which make local chromatin structure more condensed and less accessible to transcription factor binding (Jaenisch and Bird, 2003; Jones et al., 1998; Nan et al., 1998). The mammalian MBP family consists of MeCP2, MBD1, MBD2, MBD3, and MBD4. Differences in affinities of MBPs for different CpG-methylated DNA sequences may play a role in selective recruitment of MBPs to gene promoters (Fraga et al., 2003). For example, a complex of MBD2 and several NuRD chromatin remodeling proteins, initially called MeCP1, binds to DNA containing at least 12 symmetrically methylated CpGs (Meehan et al., 1989), whereas MeCP2 binds to a single methylated CpG (Ballestar and Wolffe, 2001). In the present study, we demonstrated that the nicotine treatment significantly increased the binding of MeCP2 to the CpG₋₅₂ locus at AT₂R promoter in pup brains *in vivo* in the context of intact chromatin, suggesting a novel mechanism in sequence-nonspecific CpG methylation and gene repression in the developing brain resulting from perinatal stress. Consistently, it has been demonstrated that the binding of MeCP2 at the TATA-box region may directly repel the binding of TATA-box binding protein to the TATA element (Kitazawa and Kitazawa, 2007).

Of importance, the present study provides the cause-and-effect evidence in the perinatal stress-induced increase in CpG methylation and AT₂R gene repression in the developing brain and its pathophysiological consequence of heightened HIE vulnerability in the neonate. Epigenetic states of DNA methylation are reversible. The causal effect of increased CpG₅₂ methylation in the nicotine-induced AT₂R gene repression in the brain was demonstrated with a DNA methylation inhibitor 5-aza-2'-deoxycytidine in the present study. 5-Aza-2'-deoxycytidine, *via* inhibition of DNA methyltransferase 1, has been demonstrated to cause demethylation of genes and rescue gene expressions both *in vivo* and *in vitro*, and has been widely used to inhibit DNA methylation (Alikhani-Koopaei et al., 2004; Altundag et al., 2004; Creusot et al., 1982; Jaenisch and Bird, 2003; Lin et al., 2001; Michalowsky and Jones, 1987; Pinzone et al., 2004; Richardson, 2002; Scheinbart et al., 1991; Segura-Pacheco et al., 2003; Villar-Garea et al., 2003). In the present study, we demonstrated that ICV administration of 5-aza-2'-deoxycytidine reversed the nicotine-induced CpG₅₂ methylation, rescued TBP binding and restored AT₂R mRNA and protein expression in the developing brain. In agreement to the present finding, a previous study in rats demonstrated that intraperitoneal injection of 5-aza-2'-deoxycytidine caused demethylation of 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2) gene promoter in the kidney, lung, and liver (Alikhani-Koopaei et al., 2004). These *in vivo* changes induced by 5-aza-2'-deoxycytidine were compatible with a decline in 11 β -HSD2 promoter DNA methylation in cell lines, and the decreased level of promoter methylation resulted in a higher expression of the 11 β -HSD2 gene both *in vivo* and *in vitro* (Alikhani-Koopaei et al., 2004). The ability of 5-aza-2'-deoxycytidine to rescue a gene expression in the presence of fetal stress has also been demonstrated in the developing heart showing that 5-aza-2'-deoxycytidine restores fetal stress-induced down-regulation of PKC mRNA and protein expression in fetal rat hearts (Lawrence et al., 2011; Meyer et al., 2009; Patterson et al., 2010; Xiong et al., 2010).

The finding that 5-aza-2'-deoxycytidine abrogated the nicotine-induced increase in the vulnerability of HI injury in the pup brains provides novel and causative evidence of increased promoter methylation linking perinatal stress and pathophysiological consequence of heightened HIE vulnerability in the neonate. The Rice-Vannucci model of unilateral common carotid artery ligation followed by 2.5 to 3 hours 8% oxygen treatment that produces extensive brain damage of over 30% infarction in neonatal rats has been widely used in studies of potential therapeutic interventions. However, few studies examined the brain susceptibility to mild HI injury in neonates, which may present subtle yet clinically relevant changes and require more sophisticated experimental procedures. In the present study, we used a modified Rice-Vannucci model with much shorter treatment period of pups with 8% oxygen for 1.5 hours, which produced only a mild HI insult of much reduced brain injury of about 15% infarction in control pups. As it reported previously (Li et al., 2012), this mild brain HI injury was significantly increased in nicotine-treated male pups, suggesting a critical importance of appropriate model in investigating subtle changes of heightened brain vulnerability of HIE in newborns. Although the assessment of brain injury by TTC staining in the present study may not be able to clearly distinguish the damage between neurons and white matter, it appears that infarction mainly occurs in the cortex. Of importance, the nicotine-induced increase in brain HI injury was rescued by 5-aza-2'-deoxycytidine. Future studies are needed to further evaluate the significance of this rescue by examining both short and long term effects on behavioral, motor, and cognitive functions in neonates and later in life.

The present investigation provides evidence of a novel mechanism of increased methylation of a single CpG₅₂ near the TATA element in epigenetic repression of gene expression patterns in the developing brain and the resultant increase in HIE vulnerability in neonatal brains caused by fetal and neonatal stress. Whether the effect of nicotine is specific for hypoxic-ischemic brain injury or it can be generalized remain to be determined. Although it

may be difficult to translate the present findings directly into the humans, the possibility that perinatal nicotine exposure may result in programming of a specific gene expression in the brain with a consequence of increased brain HI injury in the neonate, provides a mechanism worthy of investigation in humans. This is because maternal cigarette smoking and use of nicotine gum and patch are a major stress to the developing fetus and newborn. Of importance, the present finding that inhibition of DNA methylation rescued perinatal stress-induced programming of ischemic-sensitive phenotype in the developing brain provides a mechanistic understanding of pathophysiology of HIE and may suggest new insights in the development of therapeutic strategies in the treatment of HIE in the neonate.

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Highlights

Perinatal nicotine exposure induces promoter methylation of AT₂R gene in the developing brain.

Increased promoter methylation represses AT₂R gene expression in the brain.

Inhibition of DNA methylation restores nicotine-induced AT₂R gene repression.

Demethylating agent rescues nicotine-mediated heightened HIE vulnerability in neonatal brains.

Figure 1. Binding of TBP to TATA element at AT₂R promoter in rat pup brains

Nuclear extracts (NE) from 10-day-old pup brains were incubated with double-stranded oligonucleotide probes containing the TATA element at -48 in the absence or presence of a TBP antibody. Cold competition was performed with unlabeled competitor oligonucleotide at a 200-fold molar excess.

Figure 2. CpG₋₅₂ methylation inhibited TBP binding affinity at AT₂R promoter

The binding affinity of TBP to the TATA element was determined in competition studies performed in pooled nuclear extracts from 10-day-old pup brains with the increasing ratio of unlabelled/labelled oligonucleotides encompassing the TATA element at -48 with unmethylated (UM) or methylated (M) CpG₋₅₂ locus.

Figure 3. 5-Aza-2'-deoxycytidine abrogated nicotine-induced methylation of CpG₅₂ locus
Methylation of CpG₅₂ locus at AT₂R promoter was determined in 10-day-old pup brains isolated from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA) (1 mg/kg). Data are means $\pm$ SEM, $n = 5$. * $P < 0.05$ versus the control group.

Figure 4. 5-Aza-2'-deoxycytidine reversed nicotine-induced changes in TBP and MeCP2 binding at AT₂R promoter

MeCP2 (panel A) and TBP (panel B) binding to the TATA element at AT₂R promoter *in vivo* in the context of intact chromatin was determined with ChIP assays in 10-day-old pup brains from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA) (1 mg/kg). Data are means $\pm$ SEM, $n = 5$. * $P < 0.05$ versus the control group.

Figure 5. 5-Aza-2'-deoxycytidine restored nicotine-induced down-regulation of AT₂R mRNA and protein expression

AT₂R protein (panel A) and mRNA (panel B) abundance was determined in 10-day-old pup brains from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA) (1 mg/kg). Data are means $\pm$ SEM, $n = 5$. * $P < 0.05$ versus the control group.

Figure 6. 5-Aza-2'-deoxycytidine rescued nicotine-induced increase in neonatal brain HI injury
Hypoxic-ischemic injury was determined in 10-day-old pup brains from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA) (1 mg/kg). Data are means $\pm$ SEM, $n = 4$ to 7. * $P < 0.05$ versus the control group.