Perinatal Nicotine Exposure Increases Vulnerability of Hypoxic–Ischemic Brain Injury in Neonatal Rats
Role of Angiotensin II Receptors

Yong Li, MD; Daliao Xiao, PhD; Chiranjib Dasgupta, PhD; Fuxia Xiong, MD, PhD; Wenni Tong, PhD; Shumei Yang, PhD; Lubo Zhang, PhD

Background and Purpose—Maternal cigarette smoking increases the risk of neonatal morbidity. We tested the hypothesis that perinatal nicotine exposure causes heightened brain vulnerability to hypoxic–ischemic (HI) injury in neonatal rats through aberrant expression patterns of angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors in the developing brain.

Methods—Nicotine was administered to pregnant rats through subcutaneous osmotic minipumps. HI brain injury was determined in 10-day-old pups. AT1R and AT2R expression patterns were assessed through Western blotting, quantitative polymerase chain reaction, immunofluorescence, and confocal imaging.

Results—Perinatal nicotine exposure significantly increased HI brain infarct size in male, but not female, pups. In fetal brains, nicotine caused a decrease in mRNA and protein abundance of AT2R but not AT1R. The downregulation of AT2R persisted in brains of male pups, and nicotine treatment resulted in a significant increase in methylation of CpG locus 3 bases upstream of TATA-box at the AT2R gene promoter. In female brains, there was an increase in AT2R but a decrease in AT1R expression. Both AT1R and AT2R expressed in neurons but not in astrocytes in the cortex and hippocampus. Central application of AT1R antagonist losartan or AT2R antagonist PD123319 increased HI brain infarct size in both male and female pups. In male pups, AT2R agonist CGP42112 abrogated nicotine-induced increase in HI brain infarction. In females, PD123319 uncovered the nicotine’s effect on HI brain infarction.

Conclusion—Perinatal nicotine exposure causes epigenetic repression of the AT2R gene in the developing brain resulting in heightened brain vulnerability to HI injury in neonatal male rats in a sex-dependent manner. (Stroke. 2012;43:2483-2490.)

Key Words: AT1R/AT2R ■ hypoxic–ischemic brain injury ■ neonatal rat ■ nicotine

H ypoxic–ischemic encephalopathy (HIE) occurs in one to 6 per 1000 term newborns and causes severe mortality and long-lasting morbidity, including cerebral palsy, seizure, and cognitive retardation in infants and children.1,2 Although the underlying mechanisms of heightened brain vulnerability to hypoxic–ischemic (HI) injury in newborns remain largely elusive, recent studies suggest a possible cause of aberrant brain development due to fetal insults.3 Maternal cigarette smoking is the single most widespread perinatal insult in the world. As one of the major components of cigarette smoking, nicotine readily crosses the placenta and produces higher nicotine concentrations in the fetal circulation than that experienced by the mother.4 Epidemiological and animal 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.5,6

However, whether and to what extent perinatal nicotine exposure adversely affects the brain susceptibility to HI injury in newborns remains unknown. The present study tested the hypothesis that maternal nicotine administration during gestation results in heightened brain vulnerability to HI injury in neonatal rats. Given that the brain renin–angiotensin system plays a vital role in the development and progression of cerebrovascular diseases, and both angiotensin II type 1 (AT1R) and type 2 (AT2R) receptors are pivotal players in the pathogenesis of ischemic brain injury,7–9 we sought to investigate further the role of AT1R and AT2R in the nicotine-mediated ischemia-sensitive phenotype of neo-

Received May 14, 2012; accepted May 21, 2012.
From the Center for Perinatal Biology, Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA (Y.L., D.X., C.D., F.X., W.T., L.Z.); the Department of Chemistry and Biochemistry, California State University, San Bernardino, CA (S.Y.); and the Department of Neurology, First Affiliated Hospital of Chongqing Medical University, Chongqing, China (Y.L.).

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.112.664698/-/DC1.

Correspondence to Lubo Zhang, PhD, Center for Perinatal Biology, Division of Pharmacology, Department of Basic Sciences, Loma Linda University, School of Medicine, Loma Linda, CA 92350. E-mail lzhang@llu.edu

© 2012 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.112.664698
natal brains. We present evidence of a novel finding that perinatal nicotine exposure causes epigenetic programming of AT,R gene repression in the developing brain resulting in the increased brain susceptibility to HI injury in neonatal male rats in a sex-dependent manner and suggest new insights of molecular mechanisms linking maternal cigarette smoking to heightened HIE vulnerability in newborns.

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. On Day 21 of pregnancy, some rats were euthanized and fetal (E21) brains were isolated. Other rats were allowed to give birth, and further studies were conducted in 10-day-old neonatal (P10) pups of both sexes. All procedures and protocols allowed to give birth, and further studies were conducted in 10-day-old neonatal (P10) pups of both sexes. 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 HI Treatment and Intracerebroventricular Injection

A modified Rice-Vannucci model was conducted in P10 pups.11 Pups were anesthetized with 2% isoflurane and the right common carotid artery was ligated. After recovery for 1 hour, pups were treated with 8% O2 for 1.5 or 2.5 hours. To determine the role of AT,R and AT,R brain HI injury. AT,R antagonist losartan (Merck), AT,R antagonist PD123319 (Sigma-Aldrich), and AT,R selective agonist CPG42112 (TOCRIS Bioscience) were administered intracerebroventricularly, respectively, before the HI treatment. Pups were anesthetized and fixed on a stereotaxic apparatus (Stoeling, Wood Dale, IL). An incision was made on the skull surface and bregma was exposed. All agents were injected at a rate of 1 μL/min with a 10-μL syringe (Stoeling) on the right hemisphere following the coordinates relative to bregma: 2 mm posterior, 1.5 mm lateral, and 3.0 mm below the skull surface.12 Saline was injected as a control. The injection lasted 2 minutes and the needle was kept for additional 5 minutes before its removal. The incision was sutured.

Infarct Size Measurement

Pups were anesthetized and 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 (Sigma-Aldrich) for 5 minutes at 37°C and then fixed with 10% formaldehyde overnight. Each slice was weighed, photographed separately, and the percentage of infarction area for each slice was analyzed by Image J software (Version 1.40; National Institutes of Health, Bethesda, MD), corrected by 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 μg/mL leupeptin, and 5 μg/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 90 minutes. Proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies against AT1R (1:100) and AT2R (1:1000; Santa Cruz Biotechnology; Santa Cruz, CA) as described previously.13 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.

Real-Time Reverse Transcription–Polymerase Chain Reaction

RNA was extracted from brains and abundance of AT1R, AT1bR, and AT2R mRNA was determined by real-time reverse transcription–polymerase chain reaction using an Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously.14 The primers used were: AT1,R, 5'-ggcagaggatgcctgttga-3' (forward) and 5'-ccttgggagaggtgcatg-3' (reverse); AT1bR, 5'-tgctcaccctcctacctacctca-3' (forward) and 5'-gcacctccatcatccttgg-3' (reverse); and AT2R, 5'-caatctgctgttgaactct-3' (forward) and 5'-tcgacatacagacctgcaagaag-3' (reverse). Real-time reverse transcription–polymerase chain reaction was performed in a final volume of 25 μL. Each polymerase chain reaction mixture consisted of 600 mmol/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 μmol/L of 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 mmol/L fluorescing, and stabilizers. The following reverse transcription–polymerase chain reaction protocol was used: 42°C for 30 minutes, 95°C for 15 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 Polymerase Chain Reaction

CpG methylation at rat AT,R gene promoter was determined as previously described.10,11 Briefly, genomic DNA was isolated from brains of P10 pups 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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, and purified with 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
serum albumin for 2 hours at room temperature and incubation with the primary antibodies at 4°C overnight, tissue sections were treated with secondary antibodies raised against mouse and rabbit IgG conjugated with fluorescein isothiocyanate and Texas Red (Santa Cruz), respectively, for 2 hours at room temperature. After 3 washes, sections were stained with Hoechst 33258 (5 μg/mL; Sigma) for 1 minute. The sections were then covered with Permount reagent (Fisher) and visualized using the Zeiss LSM 710 confocal microscope, as previously described.15

**Statistical Analysis**

Data are expressed as mean±SEM. Experimental number (n) represents fetuses and 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**

**Nicotine Caused Asymmetrical Growth Restriction in Fetuses and Neonates**

Maternal nicotine administration caused a significant decrease in the body weight, but not the brain weight, in E21 fetuses, resulting in a significant increase in the brain to body weight ratio (Figure 1A). In P10 pups, both body and brain weight were decreased but the brain to body weight ratio remained significantly increased in both sexes (Figure 1B), suggesting asymmetrical growth restriction in the fetus and neonate in nicotine-treated animals.

**Nicotine Increased Brain Vulnerability to HI Injury in Male Pups**

In control animals, there was no significant difference in HI-induced brain infarct size between male and female pups (Figure 2). The nicotine treatment significantly exaggerated HI-induced brain infarct size in male, but not female, pups (Figure 2).

**Nicotine Altered Expression Patterns of AT1R and AT2R in Fetal and Neonatal Brains**

In E21 fetuses, the nicotine treatment resulted in a significant decrease in brain AT2R protein and mRNA abundance (Figure 3A–B). There was no significant effect of nicotine on AT1R protein abundance with a significant decrease in AT1aR mRNA but an increase in AT1bR mRNA abundance in the fetal brain (Figure 3B). In P10 pups, brain AT2R protein and mRNA abundance were significantly decreased in male pups...
in nicotine-treated animals (Figure 3C–D). In contrast, in female pups, nicotine caused a significant increase in brain AT2R protein and mRNA abundance (Figure 3C–D). There was no significant effect of nicotine on brain AT1R protein, AT1aR, and AT1bR mRNA abundance in male pups (Figure 3C–D). However, nicotine induced a significant reduction of brain AT1R protein and AT1aR mRNA abundance in female pups (Figure 3C–D). Immunofluorescence and confocal imaging analyses showed that both AT1R and AT2R presented in neurons but not in astrocytes in the cortex (online-only Data Supplement Figure I) and hippocampus (online-only Data Supplement Figure II) of P10 pups. It appeared that nicotine treatment increased astrocyte numbers in both cortex and hippocampus (online-only Data Supplement Figure III).

**AT1R and AT2R Protected Neonatal Rat Brains From HI Injury**

To determine the functional significance of altered AT1R and AT2R expression patterns in nicotine-induced, heightened brain vulnerability to HI injury in neonates, we first evaluated the role of AT1R and AT2R in the pathogenesis of HI brain injury in pups through intracerebroventricular (ICV) injection of AT1R or AT2R antagonists. Compared with the saline control, ICV of either losartan (Figure 4A) or PD123319 (Figure 4B) significantly increased brain infarct size in both male and female pups, suggesting that both AT1R and AT2R may be implicated in the pathogenesis of HI brain injury and confer neuroprotective properties in neonatal rat brains.

**AT2R Played a Key Role in Nicotine-Induced, Heightened Brain Vulnerability to HI Injury in Pups**

To demonstrate the cause and effect relation between nicotine-induced downregulation of brain AT2R and heightened brain vulnerability to HI injury in male pups, a selective AT2R agonist, CGP42112, was administered in male pups that had been treated with nicotine or saline control. As shown in Figure 5A, ICV administration of CGP42112 (3 μg) reversed the effect of nicotine and abrogated the difference in HI-induced brain infarct size between saline control and nicotine-treated male pups. The key role of brain AT2R in nicotine-induced heightened brain vulnerability to HI injury in neonatal rats was further tested in female pups with ICV administration of PD123319. As shown in Figure 5B, in the absence of PD123319, the nicotine treatment had no significant effect on brain HI injury in female pups. However, in the presence of PD123319 (5 μg), the effect of nicotine was uncovered and HI-induced brain infarct size was significantly increased in nicotine-treated, as compared with saline control, female pups (Figure 5B).
Nicotine Treatment Increased Methylation of CpG-52 Locus at AT2R Promoter

Recently, we have demonstrated that rat AT2R promoter has a TATA element at −48 from transcription start site, and deletion of the TATA element significantly decreases the promoter activity.13 Two CpG loci were identified at the AT2R promoter; one was located 3 bases upstream of TATA-box (CpG-52) and the other one 11 bases downstream of transcription start site (CpG11). The previous study showed that increased methylation at CpG locus 3 bases upstream of TATA-box inhibited the binding of the TATA-box binding protein and decreased promoter activity.16 As shown in Figure 6, nicotine treatment caused a significant increase in methylation of CpG-52 locus in male but not female pup brains, whereas methylation of CpG11 locus was not significantly affected.

Discussion

The new findings of the present study are: (1) perinatal nicotine exposure significantly increases brain vulnerability to HI injury in male rat pups, but not in female pups; (2) this heightened vulnerability is associated with sex-specific reprogramming of AT1R and AT2R expression patterns in the developing brain; (3) both AT1R and AT2R are implicated in the pathogenesis of HI brain injury and exhibit the neuroprotective effect in neonatal brains; (4) downregulation of AT2R in the developing brain plays a causal role in nicotine-induced, heightened brain vulnerability to HI injury in neonatal rats; and (5) increased methylation of CpG locus 3 bases upstream of TATA-box at the AT2R promoter is a mechanism of nicotine-mediated AT2R gene repression.

The present finding that perinatal nicotine exposure increased brain HI injury in neonates is novel and suggests a risk factor of maternal cigarette smoking in heightened brain HIE vulnerability in newborns. The nicotine dose used in the present study resulted in blood nicotine concentrations similar to those found in humans who smoke or use nicotine gum and patch.4,17 Nicotine readily crosses the placenta into the fetal circulation, resulting in fetal nicotine concentrations being 15% higher than maternal levels.18 It is unclear at present whether observed effects are caused by vascular effects or direct neuronal effects of nicotine. Although it may be technically challenging in measuring cerebral blood flow in neonatal rats, possible alterations in cerebral blood flow caused by nicotine treatment deserve further investigation.

The Rice-Vannucci model of unilateral common carotid artery ligation followed by 2.5 to 3 hours of 8% oxygen
treatment produces extensive brain damage in neonatal rats and is widely used in studies of potential therapeutic intervention. However, few studies examined the brain susceptibility to mild HI injury in neonates, which may present only subtle differences and require more sophisticated experimental procedures. In the present study, a shorter treatment period of pups with 8% oxygen for 1.5 hours produced mild brain damage of approximately 10% infarction in the ipsilateral hemisphere. This mild and clinically relevant brain HI injury was significantly increased by 2-fold in nicotine-treated male pups. However, the longer period of hypoxic treatment with greater brain damage in the model masked the effect of nicotine, suggesting a critical importance of appropriate model in investigating subtle changes of heightened brain vulnerability of HIE in newborns.

The growth restriction found in nicotine-treated animals presents a possible link between perinatal nicotine exposure and enhanced brain HI injury in pups given that intrauterine growth restriction is a risk factor of neonatal encephalopathy.19 Fetal hypoxia may be another possible factor enhancing the nicotine-mediated effects. Although intermittent injections of nicotine to the mother may produce episodic fetal hypoxia and a decrease in cerebral perfusion with a reduced fetal brain weight,20–22 these effects were not observed in continuous low-level infusion of nicotine through a minipump.17

The finding that ICV application of both AT1R and AT2R antagonists enhanced the severity of brain HI injury is intriguing and suggests that both AT1R and AT2R are neuroprotective in the setting of neonatal HI brain injury. Both AT1R and AT2R present in the brain with specific developmental and spatial expression patterns. In adult brains, the AT1R predominates, whereas fetal brains express high levels of AT2R that decrease during the postnatal development.23 The present study demonstrated that both AT1R and AT2R expressed exclusively in neurons in both cortex and hippocampus in neonatal rat brains, whereas AT1R expressed predominantly in astrocytes in adult brains.24 The neuroprotective effect of AT1R demonstrated in the present study is consistent with previous findings.24–26 In contrast, the present finding of a neuroprotective effect of AT2R in neonatal brains is somewhat surprising given that AT1R antagonists have been shown to exhibit neuroprotection in adult rat brains.9,27–29 These findings highlight the important differences between immature and mature brains in AT1R-mediated responses. It has been shown that apoptotic cell death is more prominent in immature brains to HI insult, but necrotic cell death is more common in adult brains in response to acute insults such as HI or excitotoxicity.30,31 Although long-term and systemic administration of AT1R antagonists often showed neuroprotective effects of the brain through multiple systemic effects, the acute and local direct effects of AT1R antagonists in modulating brain HI injury are indeed less clear and may be quite different from those seen in the long-term and systemic effects. Indeed, similar to the present finding, the previous studies demonstrated a direct adverse effect of local administration of AT1R antagonists in the setting of acute ischemic injury in the heart13,32 despite well-documented protective effects of long-term and systemic administration of AT1R blockers in preventing the deleterious consequences of ischemia and reperfusion injury and reducing cardiac remodeling.

Of importance, the present study demonstrated that perinatal nicotine exposure-mediated, heightened brain vulnerability to HI injury in male pups was associated with a significant decrease in brain AT2R expression. Additionally, the ICV administration of the AT2R agonist CGP42112 abrogated nicotine’s effect. It has been demonstrated that direct stimulation of AT2R in the brain with CGP42112 confers neuroprotective effects in a conscious rat model of stroke, which is beyond blood pressure regulation.26 These results provide evidence of a causal role of AT2R downregulation in the nicotine-induced increase in brain HI injury in the pups. Our recent study has revealed that rat AT2R promoter has a TATA element at -48 from transcription start site and deletion of the TATA-box significantly decreases the promoter activity.13 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 is intriguing and suggests an important mechanism of site-specific CpG methylation in epigenetic repression of AT2R gene in the developing brain. It has been demonstrated that increased methylation at CpG locus 3 bases upstream of TATA-box inhibits the binding of the TATA-box binding protein and decreases receptor activator of nuclear factor-κB ligand gene promoter activity.16 Unlike CpG_{-52} locus, methylation of CpG_{+11} locus was not significantly altered, suggesting its minimal role in programming of AT2R gene expression patterns in the brain. Perinatal nicotine-mediated increase in sequence-specific CpG methylation has recently been demonstrated in the Egr-1 binding site at PKCε promoter in the developing heart, which causes PKCε gene repression.10 Interestingly, nicotine had no significant effect on methylation of CpG_{-52} locus in female pup brains, demonstrating a sex-specific effect at a developmental period that
sex hormonal influences are minimal. This suggests there are transcriptional distinctions that are wired in males and females long before sex steroids are involved. Similar findings of sex-specific CpG methylation and epigenetic repression of the PKCe gene were obtained in male fetal rat hearts in response to hypoxia, in which the greater expression of estrogen receptors in female fetuses may convey a protection in stress-mediated epigenetic modifications. In the present study, the mechanism of increased AT₂R expression in female pup brains is not clear at present. A possible mechanism is that stress-mediated downregulation of glucocorticoid receptors may contribute to the upregulation of AT₂R, as shown recently in fetal rat hearts. Additionally, it has been shown that estrogen receptors mediate the downregulation of AT₁R but upregulation of AT₂R in rodents. Consistent with the present findings, sex differences in perinatal stress-mediated epigenetic programming of gene expression patterns and subsequent disease development have been well reported previously with males often being prone to be at higher risk of disease development at an earlier age than females.

The present investigation provides novel evidence that perinatal nicotine exposure increases brain susceptibility to HI injury through reprogramming of AT₁R and AT₂R expression patterns in rat pups. Although it may be difficult to translate the present findings directly into humans, the possibility that antenatal stresses may result in programming of specific gene expression patterns in the developing brain resulting in heightened vulnerability of newborn brains to HI injury provides a mechanistic understanding worthy of investigation in humans. The clinical significance of the present study is warranted because maternal cigarette smoking and use of nicotine gum and patch during gestation present a major stress to the developing fetus and because HIE in newborns causes severe mortality and long-lasting morbidity yet the underlying mechanisms remain largely elusive. Further studies on the epigenetic regulation of AT₁R and AT₂R gene expression patterns in the developing brain should provide more insights into mechanisms at the molecular level and may suggest new insights of therapeutic strategies that may be beneficial for the treatment of HIE in newborns.

Acknowledgments
A portion of this research used the Loma Linda University School of Medicine Advanced Imaging and Microscopy Core, a facility supported in part by the National Science Foundation through the Major Research Instrumentation program of the Division of Biological Infrastructure Grant No. 0923559 and the Loma Linda University School of Medicine.

Sources of Funding
This work was supported in part by the following grants: National Institutes of Health grants HL082779 (L.Z.), HL083966 (L.Z.), HL089012 (L.Z.), HL110125 (L.Z.), DA025319 (S.Y.), DA032510 (D.X.), and California Tobacco-Related Disease Research Program Award 18KT-0024 (D.X.).

Disclosures
None.

References


Perinatal Nicotine Exposure Increases Vulnerability of Hypoxic–Ischemic Brain Injury in Neonatal Rats: Role of Angiotensin II Receptors

Yong Li, Daliao Xiao, Chiranjib Dasgupta, Fuxia Xiong, Wanni Tong, Shumei Yang and Lubo Zhang

Stroke. 2012;43:2483-2490; originally published online June 26, 2012;
doi: 10.1161/STROKEAHA.112.664698

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/43/9/2483

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2012/06/26/STROKEAHA.112.664698.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/
SUPPLEMENTAL MATERIAL

Perinatal Nicotine Exposure Increases Vulnerability of Hypoxic-Ischemic Brain Injury in Neonatal Rats: Role of Angiotensin II Receptors

By

Yong Li, MD, Daliao Xiao, PhD, Chiranjib Dasgupta, PhD, Fuxia Xiong, MD, PhD, Wenni Tong, PhD, Shumei Yang, PhD, Lubo Zhang, PhD

Center for Perinatal Biology (Y.L., D.X., C.D., F.X., W.T., L.Z.), Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA; Department of Chemistry and Biochemistry (S.Y.), California State University, San Bernardino, CA; Department of Neurology (Y.L.), First Affiliated Hospital of Chongqing Medical University, Chongqing, China

Correspondence and Reprint Requests:
Lubo Zhang, PhD
Center for Perinatal Biology
Division of Pharmacology
Department of Basic Sciences
Loma Linda University
School of Medicine
Loma Linda, CA 92350
Tel: 909-558-4325
Fax: 909-558-4029
Email: lzhang@llu.edu
Figure 1. Immunoreactivity of AT₁R and AT₂R in the cortex of P10 pup brains. NeuN is visualized by FITC (green). AT₁R and AT₂R are visualized with Texas Red (red). Arrows show the peri-nuclear location of AT₁R and AT₂R. Scale bar: 50 μm. Representative immunofluorescence confocal images of samples from 3 animals in each group are shown.
## Figure 2

Immunoreactivity of AT$_1$R and AT$_2$R in the hippocampus of P10 pup brains. NeuN is visualized by FITC (green). AT$_1$R and AT$_2$R are visualized with Texas Red (red). Arrows show the peri-nuclear location of AT$_1$R and AT$_2$R. Scale bar: 50 μm. Representative immunofluorescence confocal images of samples from 3 animals in each group are shown.
Figure 3. Immunostaining of astrocytes with GFAP in the cortex (A) and hippocampus (B) of P10 pup brains. Scale bar: 50 μm. Representative immunofluorescence confocal images of samples from 3 animals in each group are shown.