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

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## Abstract

**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 *via* aberrant expression patterns of angiotensin II type 1 ($AT_1R$) and type 2 ($AT_2R$) receptors in the developing brain.

**Methods—**Nicotine was administered to pregnant rats *via* subcutaneous osmotic minipumps. HI brain injury was determined in 10-day-old pups. $AT_1R$ and $AT_2R$ expression patterns were assessed *via* Western blotting, q-PCR, 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 $AT_2R$, but not $AT_1R$. The downregulation of $AT_2R$ persisted in brains of male pups, and nicotine treatment resulted in a significant increase in methylation of CpG locus three bases upstream of TATA-box at $AT_2R$ gene promoter. In female brains, there was an increase in $AT_2R$, but a decrease in $AT_1R$ expression. Both $AT_1R$ and $AT_2R$ expressed in neurons but not in astrocytes in the cortex and hippocampus. Central application of $AT_1R$ antagonist losartan or $AT_2R$ antagonist PD123319 increased HI brain infarct size in both male and female pups. In male pups, $AT_2R$ 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 $AT_2R$ gene in the developing brain resulting in heightened brain vulnerability to HI injury in neonatal male rats in a sex-dependent manner.

### Keywords

nicotine; $AT_1R/AT_2R$; neonatal rat; hypoxic-ischemic brain injury

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**Disclosures**
None.

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## Introduction

Hypoxic-ischemic encephalopathy (HIE) occurs in 1 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 in 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 the heightened brain vulnerability to HI injury in neonatal rats. Given that brain renin-angiotensin system plays a vital role in the development and progression of cerebrovascular diseases, and both angiotensin II type 1 ($AT_1R$) and type 2 ($AT_2R$) receptors are pivotal players in the pathogenesis of ischemic brain injury,[7,8,9] we sought to investigate further the role of $AT_1R$ and $AT_2R$ in the nicotine-mediated ischemia-sensitive phenotype of neonatal brains. Herein, we present evidence of a novel finding that perinatal nicotine exposure causes epigenetic programming of $AT_2R$ 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 two groups: 1) saline control and 2) nicotine administration *via* osmotic minipumps (4 μg/kg/minute) implanted subcutaneously from day 4 of gestation to day 10 after birth.[10] 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 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 (ICV) 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% $O_2$ for 1.5 or 2.5 hours. To determine the role of $AT_1R$ and $AT_2R$ in brain HI injury, $AT_1R$ antagonist losartan (Merck), $AT_2R$ antagonist PD123319 (Sigma-Aldrich) and $AT_2R$ selective agonist CGP42112 (TOCRIS bioscience) were administered intracerebroventricularly, respectively, prior to the HI treatment. Pups were anesthetized and fixed on a stereotaxic apparatus (Stoelting, 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/minute with a 10 μl syringe (Stoelting, Wood Dale, IL) on the right hemisphere

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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 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 killed 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 (TTC; Sigma-Aldrich) for 5 minutes at 37°C and then fixed by 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 Institute 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 mM NaCl, 50 mM Tris.HCl, 10 mM EDTA, 0.1% Tween-20, 1% Triton, 0.1% β-mercaptoethanol, 0.1 mM 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% SDS and separated by electrophoresis at 100 V for 90 minutes. Proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies against $AT_1R$ (1:100) and $AT_2R$ (1:1000) (Santa Cruze 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 the Kodak ID image analysis software. Band intensities were normalized to GAPDH.

### Real-time RT-PCR

RNA was extracted from brains and abundance of $AT_{1a}R$, $AT_{1b}R$ and $AT_2R$ mRNA was determined by real-time RT-PCR using Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously.[13] The primers used were: $AT_{1a}R$, 5′-ggagaggattcgtggcttgag-3′ (forward) and 5′-ctttctgggagggttgtgtgat-3′ (reverse); $AT_{1b}R$, 5′-atgtctccagtcccctctca-3′ (forward) and 5′-tgacctcccatctccttttg-3′ (reverse); and $AT_2R$, 5′-caatctggctgtggctgactt-3′ (forward) and 5′-tgcacatcacaggtccaaaga-3′ (reverse). Real-time RT-PCR was performed in a final volume of 25 μl. Each PCR reaction mixture consisted of 600 nM of primers, 33 units of M-MLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) containing 0.625 unit Taq polymerase, 400 μM each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris-HCl, 6 mM $MgSO_4$, SYBR Green I, 20 nM fluorescing and stabilizers. The following RT-PCR 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. GAPDH was used as an internal reference and serial dilutions of the positive control was performed on each plate to create a standard curve. PCR was performed in triplicate, and threshold cycle numbers were averaged.

### Quantitative methylation-specific PCR

CpG methylation at rat $AT_2R$ gene promoter was determined as previously described.[10,14] 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,

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treated with sodium bisulfite at 55°C for 16 hours, and purified by EZ DNA Methylation-Gold Kit™ (Zymo Research), as previously described. Bisulfite-treated DNA was used as a template for real-time fluorogenic methylation-specific PCR (MSP) at $CpG_{-52}$ locus (forward primer, 5′-ttttttggaaagttggtaagtgttta-3′; reverse primer for C, 5′-ctctaatttccttcttatatattca-3′; reverse primer for $C^m$, 5′-ctctaatttccttcttatatattcg-3′) and $CpG_{+11}$ locus (forward primer, 5′-gaaggtttttagtggatag-3′; reverse primer for C, 5′-aaaaaaaactttcaattctatactca-3′; reverse primer for $C^m$, 5′-aaaaaaaactttcaattctatactcg-3′), respectively. GAPDH was used as an internal reference gene. Real-time MSP was performed using the iQ SYBR Green Supermix with iCycler real-time PCR system (Bio-Rad). Data are presented as the percent of methylation at the region of interest (methylated CpG/methylated CpG + unmethylated CpG ×100), as described previously.[10,14]

### Immunofluorescence staining and confocal imaging

Brains were fixed in formalin and processed to obtain 10 μm tissue slides. Antigens were retrieved with antigen retrieval buffer (Abcam) following heat-induced procedures. The following primary antibodies were employed: mouse anti-NeuN (Millipore); mouse anti-GFAP (Millipore); rabbit anti-$AT_1R$ (Santa Cruz); rabbit anti-$AT_2R$ (Santa Cruz). After blocking with 1% BSA 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 FITC and Texas Red (Santa Cruz), respectively, for 2 hours at room temperature. After three 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 (ANOVA) followed by Neuman-Keuls post hoc testing or Student's *t* test, where appropriate.

## Results

### Nicotine caused asymmetric 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 was decreased but the brain to body weight ratio remained significantly increased in both sexes (Figure 1B), suggesting asymmetric 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 $AT_1R$ and $AT_2R$ in fetal and neonatal brains

In E21 fetuses, the nicotine treatment resulted in a significant decrease in brain $AT_2R$ protein and mRNA abundance (Figure 3A, 3B). There was no significant effect of nicotine on $AT_1R$ protein abundance with a significant decrease in $AT_{1a}R$ mRNA but an increase in $AT_{1b}R$ mRNA abundance in the fetal brain (Figure 3B). In P10 pups, brain $AT_2R$ protein

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and mRNA abundance was significantly decreased in male pups in nicotine-treated animals (Figure 3C, 3D). In contrast, in female pups nicotine caused a significant increase in brain $AT_2R$ protein and mRNA abundance (Figure 3C, 3D). There was no significant effect of nicotine on brain $AT_1R$ protein, $AT_{1a}R$ and $AT_{1b}R$ mRNA abundance in male pups (Figure 3C, 3D). However, nicotine induced a significant reduction of brain $AT_1R$ protein and $AT_{1a}R$ mRNA abundance in female pups (Figure 3C, 3D). Immunofluorecsence and confocal imaging analyses showed that both $AT_1R$ and $AT_2R$ presented in neurons but not in astrocytes in the cortex (Supplemental Material Figure 1) and hippocampus (Supplemental Material Figure 2) of P10 pups. It appeared that nicotine treatment increased astrocyte numbers in both cortex and hippocampus (Supplemental Material Figure 3).

### $AT_1R$ and $AT_2R$ protected neonatal rat brains from HI injury

To determine the functional significance of altered $AT_1R$ and $AT_2R$ expression patterns in nicotine-induced, heightened brain vulnerability to HI injury in neonates, we firstly evaluated the role of $AT_1R$ and $AT_2R$ in the pathogenesis of HI brain injury in pups *via* intracerebroventricular (ICV) injection of $AT_1R$ or $AT_2R$ 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 $AT_1R$ and $AT_2R$ may be implicated in the pathogenesis of HI brain injury and confer neuroprotective properties in neonatal rat brains.

### $AT_2R$ 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 $AT_2R$ and heightened brain vulnerability to HI injury in male pups, a selective $AT_2R$ 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 $AT_2R$ 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 $AT_2R$ promoter

Recently, we have demonstrated that rat $AT_2R$ 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 $AT_2R$ promoter, one was located three bases upstream of TATA-box ($CpG_{-52}$) and the other one eleven bases downstream of transcription start site ($CpG_{+11}$). The previous study showed that increased methylation at CpG locus three 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 $CpG_{+11}$ 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 $AT_1R$ and $AT_2R$

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expression patterns in the developing brain; 3. both $AT_1R$ and $AT_2R$ are implicated in the pathogenesis of HI brain injury and exhibit the neuroprotective effect in neonatal brains; 4. downregulation of $AT_2R$ 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 three bases upstream of TATA-box at $AT_2R$ promoter is a mechanism of nicotine-mediated $AT_2R$ 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. While 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 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, shorter treatment period of pups with 8% oxygen for 1.5 hours produced a mild brain damage of about 10% infarction in the ipsilateral hemisphere. This mild and clinically relevant brain HI injury was significantly increased by more than two-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,[20,21,22] these effects were not observed in continuous low-level infusion of nicotine *via* a minipump.[17]

The finding that ICV application of both $AT_1R$ and $AT_2R$ antagonists enhanced the severity of brain HI injury is intriguing and suggests that both $AT_1R$ and $AT_2R$ are neuroprotective in the setting of neonatal HI brain injury. Both $AT_1R$ and $AT_2R$ present in the brain with specific developmental and spatial expression patterns. In adult brains, the $AT_1R$ predominates, while fetal brains express high levels of $AT_2R$ that decreases during the postnatal development.[23] The present study demonstrated that both $AT_1R$ and $AT_2R$ expressed exclusively in neurons in both cortex and hippocampus in neonatal rat brains, whereas $AT_1R$ expressed predominantly in astrocytes in adult brains.[24] The neuroprotective effect of $AT_2R$ demonstrated in the present study is consistent with previous findings.[24,25,26] In contrast, the present finding of neuroprotective effect of $AT_1R$ in neonatal brains is somewhat surprising, given that $AT_1R$ antagonists have been shown to exhibit neuroprotection in adult rat brains.[9,27,28,29] These findings highlight the important differences between immature and mature brains in $AT_1R$-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

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excitotoxicity.[30,31] Although long-term and systemic administration of $AT_1R$ antagonists often showed neuroprotective effects of the brain *via* multiple systemic effects, the acute and local direct effects of $AT_1R$ antagonists in modulating brain HI injury are indeed less clear and may be quite different as 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 $AT_1R$ antagonists in the setting of acute ischemic injury in the heart,[13,32] despite well documented protective effects of long-term and systemic administration of $AT_1R$ 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 $AT_2R$ expression. Additionally, the ICV administration of $AT_2R$ agonist CGP42112 abrogated the nicotine's effect. It has been demonstrated that direct stimulation of $AT_2R$ 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 $AT_2R$ downregulation in the nicotine-induced increase in brain HI injury in the pups. Our recent study has revealed that rat $AT_2R$ 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 three bases upstream of TATA-box at $AT_2R$ promoter in male pup brains is intriguing and suggests an important mechanism of site-specific CpG methylation in epigenetic repression of $AT_2R$ gene in the developing brain. It has been demonstrated that increased methylation at CpG locus three 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 $AT_2R$ 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 PKCε 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.[14] In the present study, the mechanism of increased $AT_2R$ 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 $AT_2R$, as shown recently in fetal rat hearts.[13] Additionally, it has been shown that estrogen receptors mediate the downregulation of $AT_1R$, but upregulation of $AT_2R$ in rodents.[33,34,35] 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.[36,37,38,39]

The present investigation provides novel evidence that perinatal nicotine exposure increases brain susceptibility to HI injury *via* reprogramming of $AT_1R$ and $AT_2R$ 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.

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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 $AT_1R$ and $AT_2R$ 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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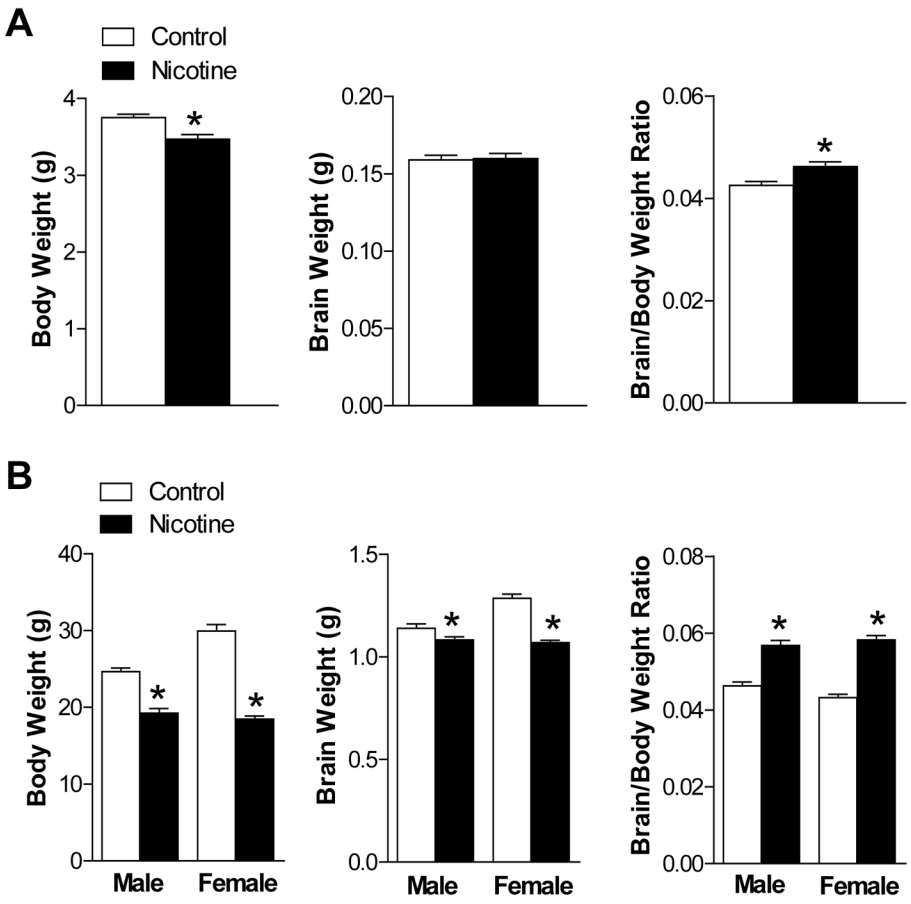


**Figure 1. Effect of nicotine on body weight, brain weight and brain to body weight ratio in E21 fetuses (Panel A; n = 27–39) and P10 pups (Panel B; n=9–13)**

Data are mean ± SEM. *P < 0.05 versus control group.

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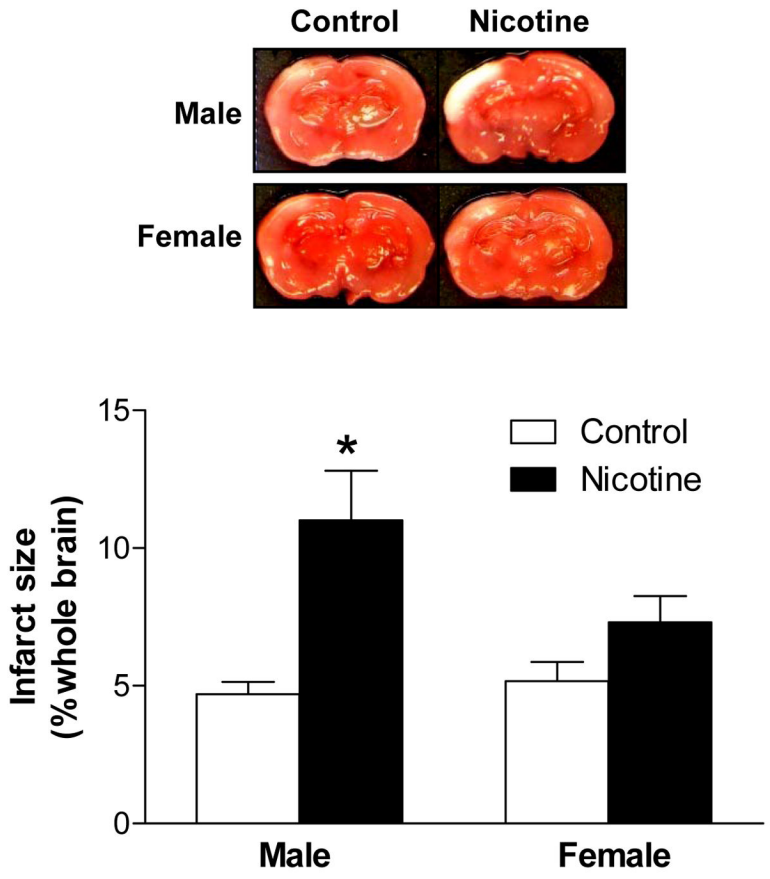


**Figure 2. Effect of nicotine on HI-induced brain infarct size in P10 pups**
Data are mean ± SEM, n = 4–6. *P < 0.05 versus control group.

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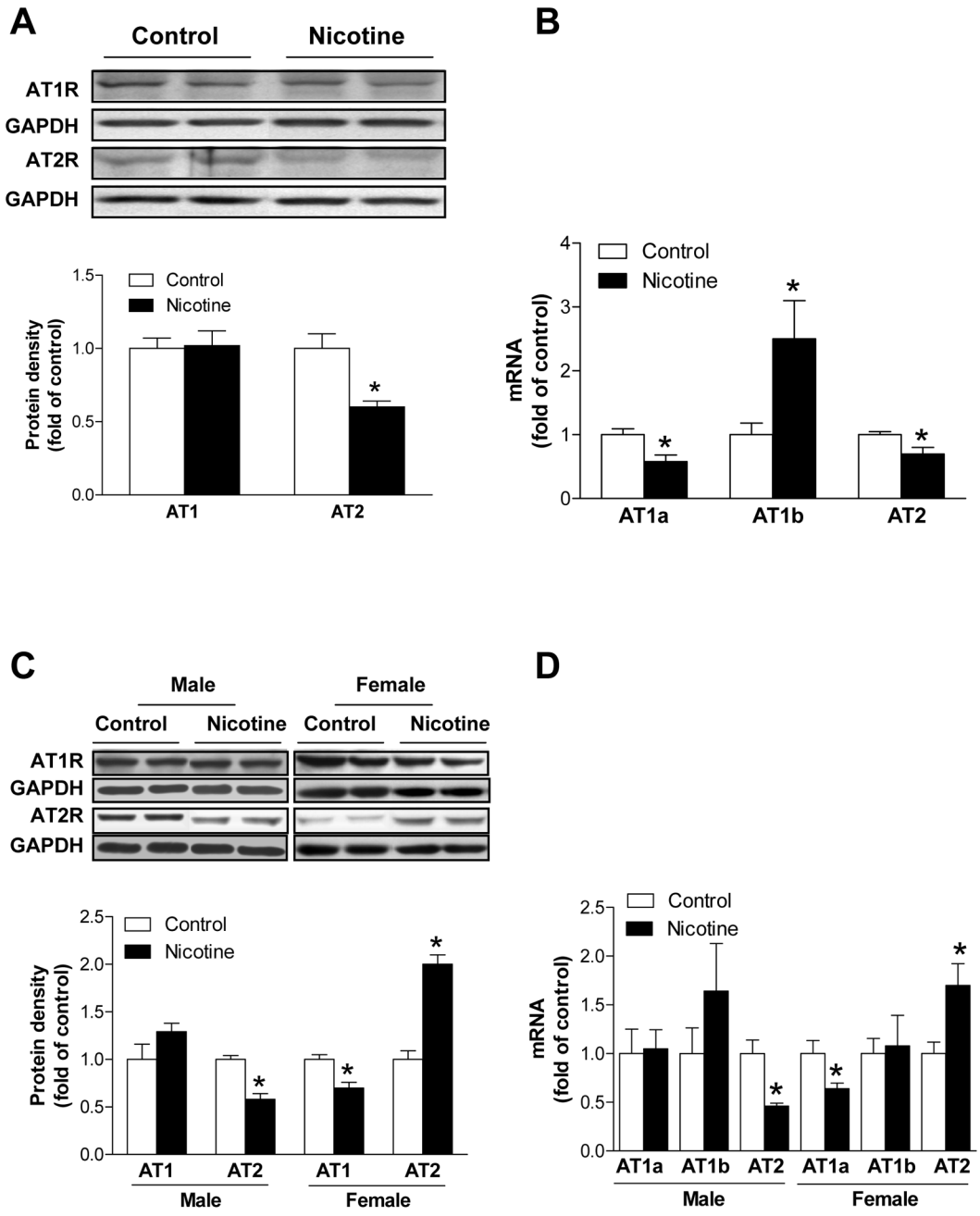


**Figure 3. Effect of nicotine on protein and mRNA abundance of $AT_1R$ and $AT_2R$ in E21 fetal (Panel A and B) and P10 pup (Panel C and D) brains**

Data are mean ± SEM, n = 4–6. *P < 0.05 versus control group.

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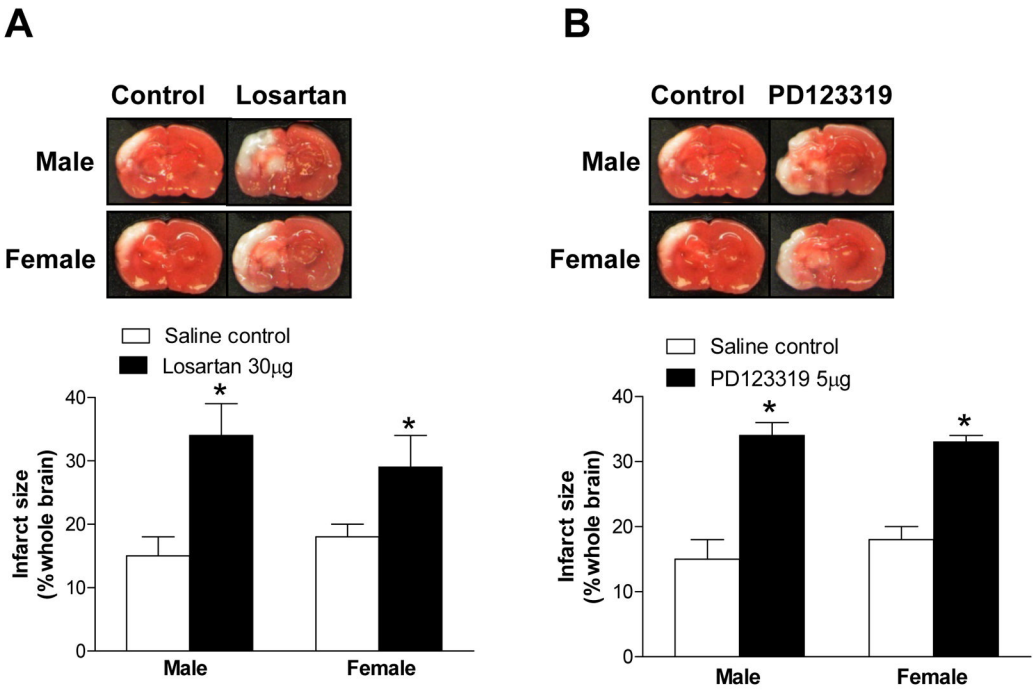


**Figure 4. Effect of losartan (Panel A) and PD123319 (Panel B) on HI-induced brain infarct size in P10 pups**

Data are mean ± SEM, n = 4–6. *P < 0.05 versus control group.

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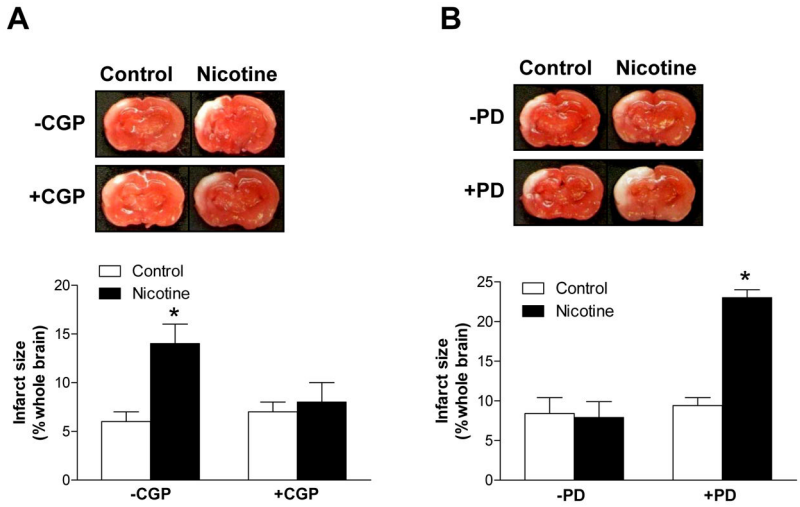


**Figure 5. Effect of CGP42112 (CGP) in male P10 pups (Panel A) and PD123319 (PD) in female P10 pups (Panel B) on nicotine-induced changes in brain HI injury**

Data are mean ± SEM, n = 4–8. *P < 0.05 versus control group.

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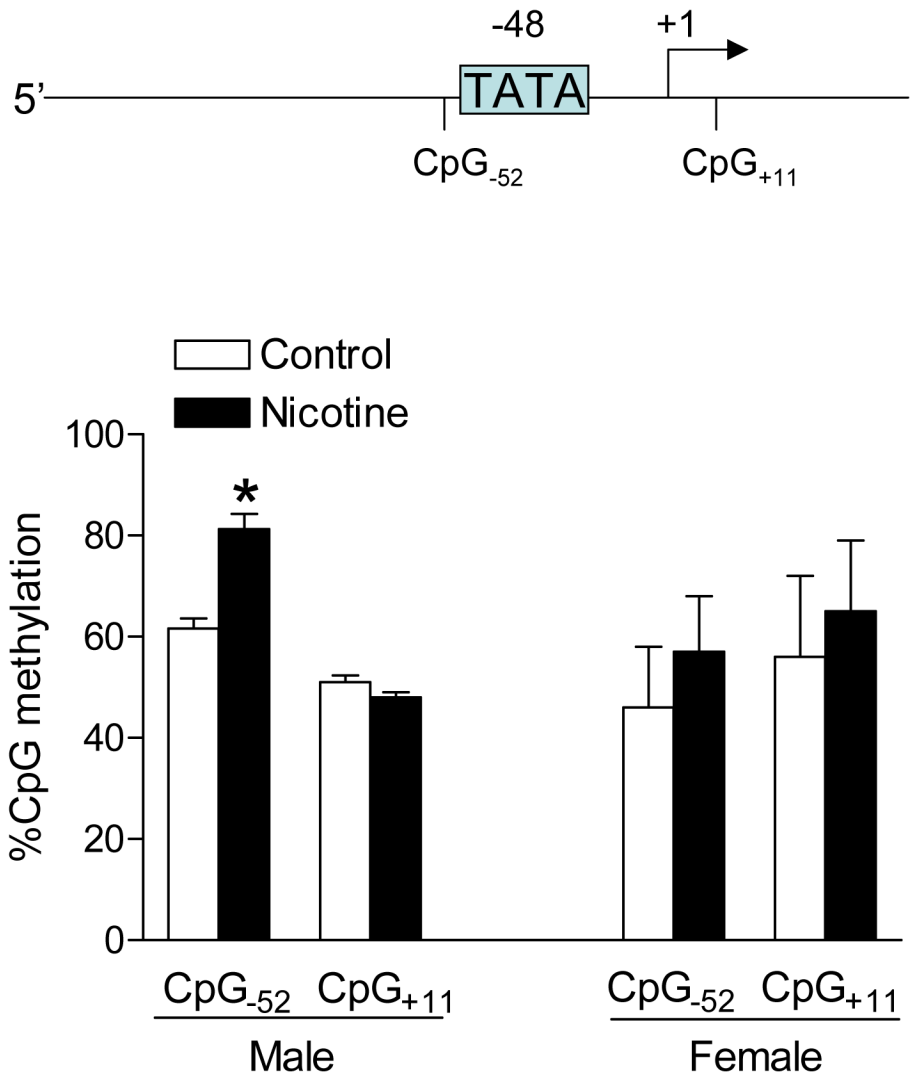


**Figure 6. Effect of nicotine on methylation of CpG loci at $AT_2R$ promoter in P10 pup brains**
Data are mean ± SEM, n = 5–10. *P < 0.05 versus control group.