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Perinatal Nicotine Exposure and Programming of HIE Sensitive Phenotype in Neonatal Rat Brains

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Perinatal Nicotine Exposure and Programming of HIE Sensitive Phenotype in Neonatal Rat Brains

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADHD	Attention-deficit/hyperactivity disorder
Ang II	Angiotensin II
AT_1R	Angiotensin II type 1 receptor
AT_2R	Angiotensin II type 2 receptor
5-Aza	5-aza-2'-oxycytidine
BBB	Blood brain barrier
BDNF	Brain derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BNIP3	Bcl-2/adenovirus E1B 19-kDa protein-binding globulin
CBF	Cerebral blood flow
CD	Conduct disorder
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
COX-2	Cyclooxygenase 2
CpG	Cytosine-phosphodiester bond-guanine
CRH	Corticotrophin-releasing hormone
DNMT1	DNA methyl transferase 1
DNMT3a	DNA methyl transferase 3a
DNMT3b	DNA methyl transferase 3b
E21	Embryonic day 21
ECM	Extracellular matrix

EEG	Electroencephalograph
Egr-1	Early growth response protein 1
EMSA	Electrophoretic mobility shift assay
EPO	Erythropoietin
ERα	Estrogen receptor α
FMR1	Fragile X mental retardation 1
FMRP	Fragile X mental retardation protein
GABA	Gamma amino butyric acid
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT-1	Glucose transporter 1
GR	Glucocorticoids receptor
GREs	Glucocorticoids response elements
HDAC	Histone deacetylase
HIE	Hypoxic-ischemic encephalopathy
HIF-1α	Hypoxia inducible factor 1 α subunit
HIF-1β	Hypoxia inducible factor 1 β subunit
HPA	Hypothalamic-pituitary-adrenal
11β-HSD1	11-beta-hydroxysteroid dehydrogenase type 1
11β-HSD2	11-beta-hydroxysteroid dehydrogenase type 2
ICV	Intracerebroventricular
IGF-2	Insulin-like growth factor 2
IUGR	Intrauterine growth restriction
LG	Licking and grooming

MBDs	Methyl-CpG-binding domain proteins
MCAO	Middle cerebral artery occlusion
MeCP2	Methyl-CpG-binding domain protein 2
miRNAs	MicroRNA
MMP-2, 3, 9	Matrix metalloproteinase 2, 3, 9
MR	Mineralocorticoid receptor
MSP	Methylation specific PCR
nAchR	Nicotinic acetylcholine receptor
ncRNAs	Non-coding RNA
NGF	Nerve growth factor
NGFI-A	Nerve growth factor-induced protein A
NMDA	N-methyl-d-aspartic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
NRT	Nicotine replacement therapy
NSE	Neuron specific enolase
P10	Postnatal day 10
P53	Protein 53
PKCε	Protein kinase C epsilon
PVN	Paraventricular nucleus
RAS	Renin-angiotensin system
RISC	RNA-induced silencing complex
ROS	Reactive oxygen species

S-100	S-100 protein
SAM	S-adenosyl-methionine
SIDS	Sudden infant death syndrome
Sp-1	Specificity protein 1
TBP	TATA-box binding protein
TSA	Trichostatin A
VEGF	Vascular endothelial growth factor

ABSTRACT OF THE DISSERTATION

Perinatal Nicotine Exposure and Programming of HIE Sensitive Phenotype in Neonatal Rat Brains

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Large epidemiological and animal studies have revealed a clear association of adverse intrauterine environment with the increased risk of metabolic, cardiovascular and neurological diseases. Maternal smoking is the single most widespread perinatal insult in the world and has been associated with adverse pregnancy outcomes for mother, fetus and the newborn. Our study aims to test the hypothesis that perinatal nicotine exposure induces reprogramming of susceptibility to hypoxic ischemic brain injury in the immature brain, focusing on the roles of AT_1R and/or AT_2R and trying to reveal the underlying epigenetic mechanisms. Therefore, we established two rat models: perinatal nicotine exposure model in time-dated pregnant rats; hypoxic-ischemic encephalopathy (HIE) in 10-day-old rat pups. In the first part of our study, we demonstrated that nicotine exposure induces aberrant brain development in P10 pups, downregulating AT_2R expression in male but upregulating AT_2R in female pup brain, enhancing brain vulnerability to HIE in a sex-specific manner. In addition, we observed both AT_1R and AT_2R are implicated in the pathogenesis of neonatal HIE and confers neuroprotective property; AT_2R plays the pivotal and causal role in nicotine induced sex-dependent alteration of vulnerability to HIE in the developing rat brain. Our further study focused on the epigenetic mechanisms involved in nicotine exposure mediated pathological effects in HIE. We demonstrated that perinatal nicotine exposure causes heightened

methylation status of a single CpG adjacent to TATA-box at AT_2R promoter, inhibiting TBP and recruiting MeCP2 binding, repressing AT_2R gene expression, contributing to the enhanced vulnerability to HIE brain injury in male rat pups. All of pathological effects are reversed by administration of 5-Aza, a well-known DNA methylase inhibitor. These findings provide new insights in understanding of the pathogenesis of HIE in newborns and may suggest potential targets for the prevention and treatment of HIE, one of the most common causes of brain damage with severe mortality and long-lasing morbidity in infants and children.

CHAPTER ONE

INTRODUCTION

Numerous epidemiological, clinical and experimental studies have shown clearly that a compromised intrauterine environment may have subtle or drastic impact on tissue/organ ontogeny, structure and function, and alter the vulnerability or resiliency to some challenges and diseases in later life (Cottrell and Seckl, 2009; Dudley et al., 2011). A wealth of evidence has indicated that an adverse fetal environment, mostly manifested as intrauterine growth restriction (IUGR), is closely associated with increased risks of development of hypertension, coronary heart disease, insulin resistance, type 2 diabetes, central obesity, hyperlipidaemia, and other neurobehavioral, neuropsychological and neuropsychiatric disorders in adulthood (Barker et al., 1993a, 2009; Dudley et al., 2011; Gluckman and Hanson, 2004; Gluckman et al., 2008; Harris and Seckl, 2011). Environmental signals can be transmitted from the mother to the fetus, impacting specific vulnerable tissues in their sensitive developmental stage, modulating normal development trajectory, remodeling their structure and function and reprogramming the resiliency or susceptibility to diseases in postnatal life (Harris and Seckl, 2011). As one of critical targets of various stressors, brain is the central organ responsible for stress responses, determining the adaptive or maladaptive responsiveness to various acute or chronic stressful conditions via making corresponding alterations in its structure and function (McEwen, 2008). The developing brain in the fetal and neonatal stage is highly plastic, flexible, and especially sensitive to numerous adverse environmental factors. Combined

with specific genetic traits, these changes of fetal and neonatal brain may contribute to high incidence of a broad spectrum of neurodevelopmental disorders in the postnatal life. Perinatal stresses, such as hypoxia, malnutrition, substances exposure and excess glucocorticoid (endogenous or exogenous), have long lasting impact on the developing brain; altering brain's ontogeny, organization, structure and function; remodeling brain's development trajectory, and reprogramming brain's vulnerability or resiliency of some neurobehavioral, neuropsychological and neuropsychiatric disorders in later life (Archer, 2011; Chen and Zhang, 2011; Chiriboga, 1998; Harris and Seckl, 2011; Seckl and Meaney, 2004; Zhang et al., 2005).

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of major causes of acute mortality as well as chronic neurological disability in newborns (Chen et al., 2009b; Vannucci, 2000). However, up to now, no universally accepted therapy is available for HIE except that a few studies implied that moderate hypothermia, administered in the early phase for full term neonates with mild or moderate encephalopathy, may reduce mortality and disability at 18 months (Perlman, 2006; Rees et al., 2011). 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 (Jensen, 2006). It is well-known that 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 (Lambers and Clark, 1996). 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). However, whether and to what extent perinatal nicotine exposure adversely affects the brain susceptibility to neonatal HI injury in newborns remains unknown. Emerging evidence has revealed that the brain renin-angiotensin system (RAS) 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, which may also implicate in neonatal HIE (Sokol et al., 2004; Schrader et al., 2005). Therefore, it is tempting to speculate that maternal stresses including perinatal nicotine exposure may impact normal brain development of fetus, specifically reprogramming some key genes expression patterns such as angiotensin II type 1 (AT_1R) and type 2 (AT_2R) receptors and contributing to alteration of the vulnerability to some challenges later in postnatal life.

Developmental Programming of Health and Disease

Fetal growth and development are a complex and dynamic process that depends on sophisticated interactions among the mother, placenta and fetus to ensure optimal growth and survival conditions (Warner and Ozanne, 2010). Environmental signals can be transmitted from the mother to the fetus, impacting specific vulnerable tissues in their sensitive developmental stage, modulating normal development trajectory, remodeling their structure and function and reprogramming the resiliency or susceptibility to diseases in postnatal life (Harris and Seckl, 2011). The hypothesis of "developmental programming of health and disease" or "fetal origins of adult disease" was put forward to

elucidate these links between adverse intrauterine environment, fetal growth and development, and disease later in life, which proposes that a wide range of environmental conditions during embryonic development and early life determine susceptibility to disease during adult life (Barker et al., 1993a; de Boo and Harding, 2006; Langley-Evans and McMullen, 2010; Seckl, 1998; Wadhwa et al., 2009; Warner and Ozanne, 2010). These programming processes may be rely on multiple factors including gestational age, duration and mode of exposure and nature of the stressor, and these processes are tissue/organ specific (Harris and Seckl, 2011). Genetic traits, epigenetic modifications and central stress mediators such as glucocorticoid or catecholamines may underpin such phenotypic plasticity. Actually, more and more evidence indicate a close link between the adverse fetal environment, mostly manifested as intrauterine growth restriction (IUGR), and increased risks of development of hypertension, coronary heart disease, insulin resistance, type 2 diabetes, central obesity, hyperlipidaemia, and other neurobehavioral, neuropsychological and neuropsychiatric disorders in adulthood (Fig. 1)(Barker et al., 1993a, 2009; Dudley et al., 2011; Gluckman and Hanson, 2004; Gluckman et al., 2008; Harris and Seckl, 2011).

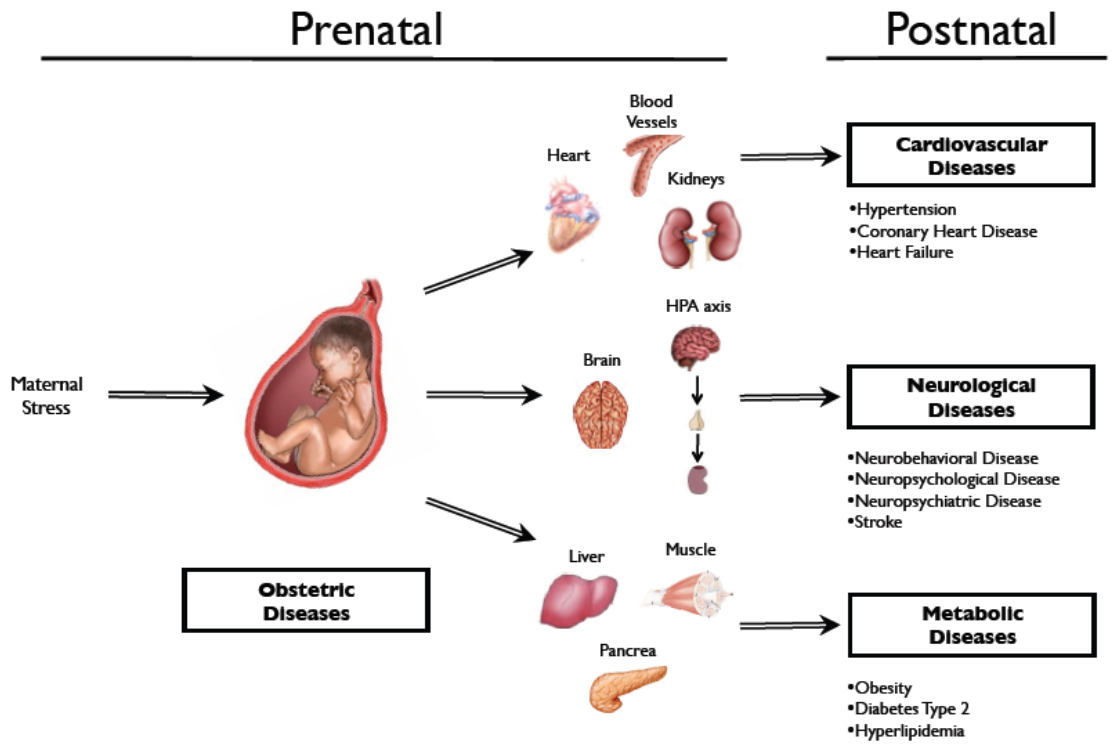

Figure 1. Developmental programming of health and disease. Maternal stress impacts normal fetal tissues/organs development and increases the risk of development of cardiovascular, metabolic syndrome, stroke and various neurobehavioral, neuropsychological, neuropsychiatric diseases later in life. HPA, hypothalamic-pituitary-adrenal.

Potential Mechanisms of Fetal Stress-Mediated Programming

Brain development consists of a series of progressive and regressive events tightly regulated by the interaction between cellular and environmental factors. Fetal brain development is especially susceptible to environmental perturbations. Prenatal stress exposure affects various neurotransmitters, neuromodulators, neurotrophic factors and cell adhesion molecules, etc., at specifically susceptible stages to alter neuronal development via both acute and chronic effects on cellular behavior and gene expression patterns (Levitt, 1998). Aberrant cellular behavior and gene expression confer permanent structure remodeling and function reprogramming, which may lead the brain to be more vulnerable to later challenges.

Intrauterine programming of the HPA axis may be one of the key common mechanisms underpinning prenatal stress and increased risk of diseases later in life (Fig. 2). Fetal HPA axis is highly susceptible to programming actions during development. Prenatal stress, such as nicotine, cocaine, alcohol, hypoxia, malnutrition and glucocorticoid, can directly or indirectly alter the set point of the HPA axis, and enhance the activity of the HPA axis in basal and stressful conditions throughout life. Glucocorticoid plays the pivotal role in such programming processes, which may be also associated with other stress mediators, such as catecholamines (Lee et al., 2008). Structures of the limbic system, including hippocampus, hypothalamus, anterior pituitary and amygdala, express high levels of GR, constituting the major target of endogenous or exogenous glucocorticoid in the brain. These GRs, particularly in hippocampus, exert crucial negative feedback regulation on the activity of the HPA axis. Maternal stress or synthetic glucocorticoid administration result in high levels of glucocorticoid exposure

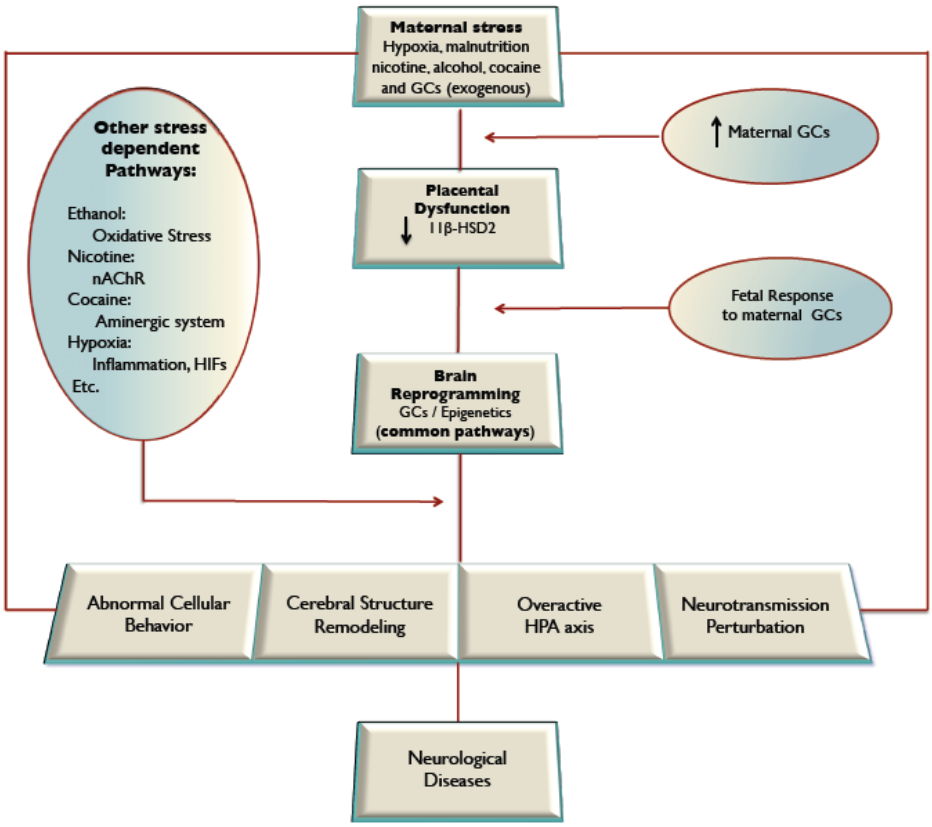

Figure 2. Mechanisms of developmental programming of neurological diseases. Prenatal stress changes normal brain developmental trajectory, alters brain cellular behavior, remodels cerebral structure and morphology, reconstructs the HPA axis activity, disturbs neurotransmission, and reprograms the vulnerability or resiliency to neurological diseases in later life, of which epigenetic modifications of glucocorticoid receptors (GRs) gene expression patterns may represent as a common pathway in response to different adverse intrauterine stimuli. 11β-HSD2, 11-beta-hydroxysteroid dehydrogenase type-2; HIFs, hypoxia inducible factors; HPA, hypothalamic-pituitary-adrenal; nAChR, nicotinic acetylcholine receptor.

to the fetus, leading to down-regulation of GR in hippocampus and attenuation of negative feedback of the HPA axis and enhancement of the HPA axis activity. The overall effects of programming allow the body being exposed to sustained elevated endogenous glucocorticoid in both basal and stress conditions, resulting in alteration of behavior, cognition, learning, memory, emotion and predisposing the individual to a variety of cardiovascular and metabolic syndromes in later life (Harris and Seckl, 2011). The programming effects of HPA function by prenatal stress may be correlated to the exposure's severity, time, duration, and genetic factors (Harris and Seckl, 2011).

Glucocorticoid is extraordinary hormone with numerous effects affecting many vital organs/systems, including the brain, heart and kidney, etc., and may regulate expression patterns of approximately 10% of human genes (Buckingham, 2006). Glucocorticoid is essential for life and plays a crucial role in the regulation of growth and development, but also is implicated in various pathogenesis. There are two major endogenous glucocorticoids, cortisol and corticosterone, both synthesized in mammalian species but with different distribution predominance between species. Cortisol is predominant in humans while its counterpart corticosterone is principally produced in rodents. As the key mediator of stress responses, glucocorticoid is mainly synthesized from cholesterol in cells of zona fasciculate of adrenal cortex (Buckingham, 2006). In normal conditions, glucocorticoid levels are strictly regulated by negative feedback of glucocorticoid on the HPA axis. Disturbance of such feedback regulation loop may result in maladaptive impacts on the brain and other organs, contributing to numerous pathophysiological changes throughout life.

Glucocorticoid affects fetal brain development mainly via interaction with its

receptors, GR and MR. GR and MR are highly expressed in the developing brain with dynamic and complicated ontogeny. During fetal development, GR is present from the early embryonic stage in most tissues, but expression of MR is relatively limited and presents during later stages of development (Holmes et al., 2003). MR is responsible for mediating effects of very low concentrations of glucocorticoid usually in physiological conditions, and GR mediates effects of relatively high levels of glucocorticoid when MR has been saturated, especially in stress response (Buckingham, 2006). Synthetic glucocorticoid is relatively selective for GR. Therefore, GR is likely to be the major player in glucocorticoid overexposure. After processes such as ligand binding, dimerization and phosphorylation, the glucocorticoid receptor-ligand complex translocates into the nucleus and binds to various GREs (glucocorticoid response elements) in the gene promoter region, resulting in activation or repression of target gene expression.

Glucocorticoid exerts effects at cellular and molecular levels to affect tissue/organ growth and differentiation. During fetal development, high levels of glucocorticoid exposure change the expression patterns of various receptors, enzymes, ion channels and transporters in most of cell types as well as alteration of various growth factors, cytoarchitecture proteins, binding proteins and other essential components of the intracellular signaling pathways (Fowden and Forhead, 2004). Such changes significantly impact the basal cellular functions and their responses to numerous stimuli, contributing to alteration of cell size, number, proliferation rate and terminal differentiation. Indirectly, glucocorticoid can also affect tissue proliferation and differentiation via altering cellular secretion of proteins, hormones, growth factors and metabolites, which

can greatly amplify its programming effects on fetal development (Fowden and Forhead, 2004). At the molecular level, glucocorticoid regulates target gene transcription, mRNA stability, translation/post-translation modifications, etc., which may be mediated by directly controlling via GREs in promoter regions of responsive genes or indirectly via other transcription factors or glucocorticoid dependent hormones (Fowden and Forhead, 2004). All of these changes induced by glucocorticoid will confer an integration of function at the system level, suggesting that glucocorticoid-mediated programming may result in dynamic, multifaceted, co-ordinated and interdependent changes in different tissues (Fowden and Forhead, 2004).

Sufficient glucocorticoid is vital for normal maturation in most regions of the developing CNS. However, during vulnerable stages of development, inappropriate levels of glucocorticoid may remodel developmental trajectories of specific brain structures and alter corresponding functions accompanied by long-lasting adverse consequences, notably disturbance of behavior, cognition and disease susceptibility in later life (Harris and Seckl, 2011; Seckl and Meaney, 2004). For example, prenatal glucocorticoid administration reduces brain weight at birth, delays myelination of the corpus callosum, retards astrocyte and vasculature maturation in sheep, and decreases cortex convolutions index and surface area in humans (Antonow-Schlorke et al., 2009; Modi et al., 2001). Prenatal stress can also diminish dendritic spine density in the anterior cingulated gyrus and orbitofrontal cortex in rats (Murmu et al., 2006). Studies in both humans and animals have revealed that hippocampus is a highly vulnerable structure particularly sensitive to prenatal glucocorticoid exposure, leading to variable memory and behavior deficits. For example, prenatal stress in rats can reduce synaptic spine density in hippocampus, which

is associated with impairment of reversal learning (Hayashi et al., 1998). Betamethasone administration in fetal baboons inhibits neurogenesis and impairs neuronal plasticity via downregulation of critical proteins such as cytoskeletal microtubule associated proteins and synaptophysin, resulting in cognition deficits (Antonow-Schlorke et al., 2003). In addition, antenatal administration of dexamethasone results in neuronal degeneration in the hippocampus subfields and reduces the hippocampus volume in a dose-dependent manner (Uno et al., 1990). It seems that chronic low levels of glucocorticoid exposure may be more deleterious than its short, sharp impact on fetal brain development (Harris and Seckl, 2011). Thus, the alteration of hippocampus structure and function may offer a plausible neuroanatomical basis for the programming effects of glucocorticoid on cognitive ability, behavior and the risk of psychological and psychiatric disorders in later life.

Long-term prenatal glucocorticoid exposure may permanently alter the "set point" and sensitivity of endocrine axis, such as the somatotrophic and hypothalamic-pituitary-adrenal axes (Fowden and Forhead, 2004; Meaney et al., 2007). The HPA axis is an important programming target in the brain. It is strictly controlled by a negative-feedback mechanism in which glucocorticoid from peripheral adrenal cortex interacts with GR in hippocampus, hypothalamus and pituitary to modulate its final level and activity of HPA axis in stress. Maternal malnutrition, inhibition of 11β- HSD2 and other prenatal stress may reduce tissue-specific expression of GR, particularly in hippocampus and impair the negative feedback regulation of glucocorticoid, thus altering the "set point" of the HPA axis (Harris and Seckl, 2011; Meaney et al., 2007; Seckl and Meaney, 2004). A large variety of animal studies have shown prenatal glucocorticoid exposure permanently

increases basal corticosterone/cortisol levels in plasma and enhances the activity of HPA axis in adult rats, sheep, guinea pigs and primates (Hawkins et al., 2000; Levitt et al., 1996; Seckl and Meaney, 2004; Uno et al., 1994). Such changes are dependent on gestational age of exposure and also show sex-specific features. Prenatal dexamethasone exposure also stimulates CRH expression in the paraventricular nucleus of hypothalamus (PVN) and in central nucleus of the amygdala, increasing corticosterone and ACTH levels in rat offspring (Levitt et al., 1996). Additionally, prenatal stress may heighten the vulnerability of CRH neuron in PVN and also program the development of the HPA axis (Tobe et al., 2005). HPA programming may be a common pathway shared by other prenatal challenges. Furthermore, prenatal glucocorticoid exposure may have effects beyond the CNS and elevate 11β-HSD1 levels in hepatic, visceral adipose tissues, which regenerates more active glucocorticoid from its inactive metabolites and further enhances adverse effects of glucocorticoid on the developing brain (Cleasby et al., 2003; Nyirenda et al., 2009). Given its wide spectrum of physiological and pathophysiological functions, it is predictable that chronic excess of glucocorticoid during fetal development and overactivity of the HPA axis may increase risks of development of hypertension, hyperglycemia, obesity, other metabocardiovascular syndrome, stroke, cognitive impairment, affective and other neuropsychiatric disorders in later life, similar to what is expected in Cushing's syndrome (Harris and Seckl, 2011).

Excessive glucocorticoid exposure may also lead to reprogramming of offspring behavior in postnatal life. Glucocorticoid can reprogram expression patterns of several key molecules implicated in the regulation of neuronal development, HPA axis and other higher cerebral functions (Drake et al., 2007). For example, prenatal glucocorticoid

exposure increases CRH and GR expression in the amygdala, a central structure mediating emotional response such as fear and anxiety (Welberg et al., 2000, 2001). Through elevated CRH and GR, amygdala may positively drive the HPA axis activity, which has been supported by transgenic study in mice (Tronche et al., 1999). Prenatal glucocorticoid exposure can also influence the development of dopaminergic system, contributing to the development of schizoaffective, attention-deficit hyperactivity, extrapyramidal disorders and drug addiction (Drake et al., 2007). Some studies also suggest that prenatal dexamethasone treatment may enhance vulnerability of cholinergic neurons to toxic challenges in later life (Diaz et al., 1995). Human studies have revealed the correlation between stressful events during the second trimester of pregnancy and incidence of schizophrenia. Of importance, such programming effects are also time-dependent (Koenig et al., 2002).

Given its deep involvement in various acute and chronic stress responses, it is easy to assume the critical position of catecholamines in programming of fetal development by prenatal stress. However, up to now, only a few studies are available to indicate the effects of catecholamines (norepinephrine and epinephrine) in fetal stress-mediated programming of the developing brain. Predictably, prenatal stress can evoke enhanced maternal release of norepinephrine via activation of the sympathetic-adrenalmedullary system, resulting in significant maternal vasoconstriction and/or disturbance of maternal cardiovascular function. This will lead to compromised delivery of oxygen and nutrients to the fetus and exaggerate adverse effects of other stress stimuli on the fetus. More importantly, Sarkar et al. (2001) reported that both norepinephrine and epinephrine rapidly repressed 11β-HSD2 mRNA expression in early and late gestational

human trophoblast cell lines, which might increase exposure levels of glucocorticoid to the fetus in uterus. The downregulation of 11β-HSD2 by catecholamines is mainly mediated by activation of α_1 and α_2 adrenoreceptors and is not dependent on β-adrenergic stimulation. However, no similar studies in vivo have been reported yet. There are studies implying that catecholamines may exert programming effects on the HPA axis in offspring of a fetal ethanol exposure model (Lee et al., 2008). However, most of these studies only confer indirect evidence of programming effects of catecholamines. Notably, some studies have indicated that maternal catecholamines can cross the placenta, and catecholamines are released by the fetus of later stage in stress (Morgan et al., 1972; Thomas et al., 1995), suggesting it is plausible that catecholamines may exert direct programming effects on fetal brain and other organ development via interaction with their specific regionally expressed α and/or β adrenoceptors. Indeed, a recent study in pregnant rats demonstrated a key role of increased norepinephrine in nicotine-mediated promoter methylation and PKC_ε gene repression in the developing heart and its sustained effect on heightened cardiac vulnerability to ischemic and reperfusion injury in adult offspring (Lawrence et al., 2011).

Perinatal Nicotine Exposure and Neurological Diseases

Although the negative effects of cigarette smoking on the development of the fetus and the newborn are well-known, it is estimated approximately 22% of mothers and 45% of fathers continue to smoke during the time of their children's birth (Nelson and Taylor, 2001). Recent studies revealed there are 250 million female smokers around the world and over 700,000 children born with exposure to cigarette smoking each year in

the United States (Pauly and Slotkin, 2008). Thus, cigarette smoking may represent the single largest modifiable neuropharmacological exposure for the fetus and newborn (Wickstrom, 2007). Currently, nicotine replacement therapy (NRT) is recommended by some obstetricians to help women to quit smoking during pregnancy although there are serious concerns about its effectiviness and safety to the mother and fetus (Pauly and Slotkin, 2008; Wickstrom, 2007).

Tobacco contains more than 4000 chemicals, including carbon monoxide, cyanide, etc., of which nicotine is the major compound with neurotoxicity (Dwyer et al., 2009). Nicotine can easily cross the placental barrier and concentrate in fetal circulation, brain, amniotic fluid and even breast milk during lactation (Wickstrom, 2007). Large amount of nicotine may induce poor nutritional status of mothers via its anorexigenic effect and compromise blood flow to the placenta through enhanced release of catecholamine from adrenals and sympathetic nerve terminals, which may also contribute to chronic placenta insufficiency. More importantly, nicotine can directly affect fetal developmental patterns through inappropriate activation of nicotinic acetylcholine receptors (nAChRs) and triggering the release of acetylcholine. The most abundant subtypes of nAChR in vertebrate brain are $\alpha4\beta2$ and $\alpha7$, of which $\alpha7$ is highly expressed in the immature brain and implicated in the response to brain injury and inflammation and participates in regulating the rate of apoptosis (Pauly and Slotkin, 2008). Acetylcholine acts as a neurotrophic factor in brain development and is involved in cell proliferation, cell differentiation, survival, apoptosis, neuritic outgrowth, neuronal migration, synaptogenesis, and establishment of neuronal circuitry and modulation of other neurotransmitters releasing. Inappropriate premature stimulation of nAChRs during

fetal development may disrupt normal prescheduled program of time and/or intensity of these neurotrophic effects and induce abnormal brain development with long-term consequences.

Ample human studies have revealed nicotine exposure during pregnancy is associated with a spectrum of adverse fetal and obstetrical outcomes: spontaneous abortion, placenta previa, placental abruption, preterm birth, stillbirth, fetal growth restriction, low birth weight, and, more severely, sudden infant death syndrome (SIDS) (Archer, 2011; Bruin et al., 2010; Eppolito and Smith, 2006; Slotkin, 1998). Epidemiological, clinical and experimental studies indicate that adverse effects of perinatal nicotine exposure are far beyond the pregnancy outcomes and neonatal morbidity or mortality. Long-term poor neurodevelopmental consequences of perinatal nicotine exposure constitute the greatest impact on society. A large amount of evidence suggests that nicotine plays a key role in mediation of long-term developmental deficits resulting from maternal smoking. Except for acetylcholine, directly or indirectly, nicotine can also affect a multitude of neurotransmitters' synthesis, release, reuptake and turnover; modulating neural proliferation, differentiation, migration and apoptosis, etc.; altering brain structure, organization and morphology; disrupting normal brain development, which finally contribute to heightened vulnerability to various neurobehavioral, neuropsychological and neuropsychiatric disorders in postnatal life (Bruin et al., 2010; Dwyer et al., 2008, 2009; Ernst et al., 2001; Pauly and Slotkin, 2008; Wickstrom, 2007).

Growing epidemiological studies have revealed that prenatal nicotine exposure is associated with various levels of motor and sensory deficits, high incidence of externalizing behavioral problems (such as oppositional, aggressive, overactive),

increased risk of attention-deficit/hyperactivity disorder (ADHD) and conduct disorder (CD), cognitive function impairment in memory, attention and learning, and the risk for developing drug dependence (e.g., nicotine, cocaine) (Eppolito and Smith, 2006; Ernst et al., 2001; Wickstrom, 2007). Consistently, animal studies with prenatal nicotine exposure also demonstrate similar presentations including hyperactivity, cognitive and somatosensory impairment, exaggerated anxiety, neurochemical imbalance, nicotine self-administration, reduction of neural cell survival and aberrant synaptogenesis (Dwyer et al., 2008, 2009; Eppolito and Smith, 2006; Levitt, 1998). These detrimental brain effects can also be induced without apparent birth weight reduction, a crude marker of poor intrauterine environment, implying the threshold of brain damage by nicotine is much lower than that of inducing IUGR (Slotkin, 1998). It is presumable that perinatal nicotine exposure may also be associated with pathogenesis of cerebral ischemia, such as neonatal HIE, in offspring.

Another problem deserving concern is the NRT during pregnancy. Recently, the NRT is widely accepted and recommended to pregnant smokers although there is a lack of convincing solid evidence for its efficacy and safety. Pharmacologically, the plasma half-life of nicotine is about 2 h (Wickstrom, 2007). However, nicotine is metabolized more quickly during pregnancy, which indicates that higher doses of NRT may be needed to attain an effect for cessation of smoking (Wickstrom, 2007). In addition, most formulations of NRT deliver nicotine continuously compared to episodic smoking in smokers. The total exposure dosage of nicotine by NRT may actually exceed those of pregnant women with mild or moderate cigarette smoking (Wickstrom et al., 2002). Furthermore, given the fact of lower threshold for altering brain development by nicotine,

more extensive studies should be conducted to justify the efficacy and safety of NRT to the fetus before its continued application in pregnancy (Pauly and Slotkin, 2008; Wickstrom, 2007).

Hypoxic Ischemic Encephalopathy (HIE)

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of major causes of acute mortality as well as chronic neurological disability in newborns (Chen et al., 2009b; Vannucci, 2000). Severe HIE disrupts normal brain development, leading to a wide variety of neurodevelopmental deficits presented as various motor and sensory abnormality, learning disability, mental retardation and seizure attacks (Vannucci, 1990; Vexler and Ferriero, 2001). The incidence of asphyxia is approximately 20% in full-term infants and up to 60% in premature infants with low birth weight, of which 20 - 50% asphyxiated infants showing HIE symptoms and signs will die and up to 25% of survivors demonstrate permanent neurological deficits such as cerebral palsy, mental retardation, learning disability and epilepsy (Perlman, 2006; Vannucci, 2000).

Compromised cerebral blood flow (CBF) is the dominant pathogenetic mechanism for neuropathophysiology due to hypoxia-ischemia, which may arise from acute reduced materno/feto-placental blood flow or from chronically compromised fetal oxygen and energy supply (Perlman, 2006; Terzidou and Bennett, 2001). The resulting patterns of HIE injury consist of periventricular white matter lesions in preterm newborn; corticosubcortical lesions, particularly in the sensomotor cortex, parasagittal region, and deep gray matter lesions of basal ganglia and thalamus in near-term and term newborns. Such patterns of injury are associated with brain maturation stage and nature of hypoxic-

ischemic injury. The etiology of brain damage secondary to HIE is complicated and multifaceted. It is well documented that energy failure due to reduction of CBF and oxygen delivery initiates the principal pathways contributing to brain cell death. In acute phase, energy depletion (primary energy failure) results in increased neuronal release of glutamate and reduced reuptake of glutamate by astrocyte, lactate acidosis, glutamate receptor (NMDA) activation, intracellular calcium accumulation, generation of ROS, lipid peroxidation, NO formation and neurotoxicity, disruption of cell essential components, and immediate or delayed cell death. Typically, about 6 - 48 h later, a second phase of injury (secondary energy failure) ensues. During this phase, accumulated mitochondrial dysfunction secondary to extended injury from primary insults (calcium influx, excitotoxicity, oxygen free radicals or NO nitrosative stress) leads to release of various cytotoxic enzymes and pro-apoptotic proteins from mitochondria causing delayed cell death (Perlman, 2006; Rees et al., 2008, 2011; Vexler and Ferriero, 2001). Evidence suggests that some circulatory and endogenous inflammatory cells/mediators may also contribute to such ongoing brain injury (Palmer, 1995; Perlman, 2006).

Of importance, compared with the adult brain, the neonatal brain shows some differences in physiological structure organization, ontogeny, function, cellular composition and signaling pathway related to gene and protein expression, demonstrating more sensitive and plastic features to challenges (Chen et al., 2009b). Such features determine that its response to brain injury is also significantly different from the adult brain, resulting in distinct acute and chronic neurological consequences, which deserves a careful consideration in experimental and clinical studies. For example, the neonatal brain shows more permeable immature blood-brain barrier (BBB) that allows readily

cross of various solutes and small insoluble molecules in blood (Chen et al., 2009b). The major responses to injury and cell death mechanisms are different in the neonatal brain, favoring more apoptotic features (Vexler and Ferriero, 2001). Additionally, the response to the treatment in the neonatal brain may be also different from that in the adult brain. In general, compared with the adult brain, the neonatal brain is more resistant to HI damage (Vannucci and Hagberg, 2004).

Up to now, no universally definite effective therapy is available to intervene with this severe neonatal encephalopathy. The only accepted therapy for HIE in clinical practice is moderate hypothermia utilized at earlier phase. A recent meta-analysis of 10 randomized controlled trials confirmed the neuroprotective effects of moderate hypothermia administered within 6 h after birth for full-term newborns with mild or moderate HIE, showing reduced mortality and neurological deficits at 18 months of age (Edwards et al., 2010; Rees et al., 2011). However, it does not improve mortality and neurological outcomes in neonates with severe HI brain injury and is contraindicated in pre-term neonates. Furthermore, the narrow administration time window also greatly restricts its clinical application. Other intervention strategies, such as application of excitatory amino acid antagonists, oxygen free radical inhibitors and scavengers, inhibition of nitric oxide formation, blockade of apoptosis cascades, application of growth factors and neurosteroids, are either still in the experimental stage or in early, ongoing, small scale clinical studies or have already failed in clinical trials, showing the lack of solid evidence to justify their extensive application (Perlman, 2006; Rees et al., 2011).

AT_1R and AT_2R in Neurological Diseases

It is well accepted that RAS is both a circulating and tissue/organ specific hormonal system implicated in various physiological and pathophysiological processes via the major peptide angiotensin II (Ang II) stimulating its specific AT_1R and AT_2R, which demonstrate opposite effects in many conditions (Sokol et al., 2004; Dasgupta and Zhang, 2011; Shi et al., 2010). Both AT_1R and AT_2R are present in the brain with different expression patterns and signaling pathways during different developmental stages. Clinical trials and experimental studies have indicated that RAS plays an important role in the development and progression of cerebrovascular diseases, but most of these studies were conducted in the mature brains. For example, clinical trials such as LIFE and MOSES have demonstrated that chronic blockade of RAS can offer neuroprotection with the prevention of first or recurrent stroke in high-risk populations, independent of its blood pressure-lowering effects (Lindholm et al., 2002; Schrader et al., 2005). Numerous studies have revealed that AT_1R antagonists exhibit anti-apoptotic, anti-inflammatory, anti-oxidant effects and improve cerebral perfusion, demonstrating vascular-dependent and -independent neuroprotection in acute stroke (Ando et al., 2004; Dai et al., 1999; Lou et al., 2004; Zhou et al., 2005). Less is known about the role of AT_2R in neurological pathophysiology. Emerging evidence indicates that AT_2R also confers beneficial effects in a variety of pathologies including various neurological disorders. Some studies reported that AT_2R was up-regulated in stroke, particularly in the ischemic area of the brain, implying its potential role in neuroprotection (Mogi et al., 2006). Increased activation of AT_2R may be responsible for some neuroprotective effects of AT_1R antagonism (Li et al., 2005). *In vitro* stimulation of AT_2R promotes intense

neurite outgrowth, which can be antagonized by PD123319 (Laflamme et al., 1996). Moreover, McCarthy et al. (2009) demonstrated centrally direct stimulation of AT_2R with CGP42112 conferred a neuroprotective role in a conscious rat model of stroke, which was beyond blood pressure regulation. The underlying mechanisms of AT_2R in neuroprotection remain to be elucidated. Some studies indicate that it may be related to its complicated interaction with AT_1R in apoptotic modulation, neuronal regeneration and vasodilation in ischemic regions following stroke (Jones et al., 2008; Saavedra et al., 2006).

Stressful stimuli in pre- or perinatal developmental stages may reprogram the expression patterns of AT_1R and AT_2R in vital organs such as the heart, vasculature, kidney and brain, contributing to later pathologies. Such programming effects may be glucocorticoids dependent and sometimes with sex diversity and involve complex epigenetic mechanisms. For example, nicotine exposure alters expression patterns of AT_1R and AT_2R in the kidney and vessels, and enhances vascular response to vasoconstrictors, which maybe contribute to development of hypertension in adulthood (Mao et al., 2009a, b; Xiao et al., 2007, 2008, 2011). In addition, maternal hypoxia during gestation can downregulate glucocorticoid receptors in the heart of fetuses and offspring and decrease GR binding to the GREs at the AT_2R promoter region, resulting in increased expression of AT_2R and heightened cardiac susceptibility to ischemic-reperfusion injury in adult offspring (Xue et al., 2011).

Epigenetics in Neurological Diseases

One of the important adaptive mechanisms that the human body could be evoked

to react to some adverse environments is through epigenetic modification of gene expression patterns. The fetal developmental stage is the most critical period for the human being because in the uterus the fetus could be exposed to inadequate or inappropriate environments that could be chemical/nutritional or non-chemical, in which different events occur in a way to induce repression or activation of gene transcription via epigenetic mechanisms (Chen and Zhang, 2011). These epigenetic changes could be associated with conditions or diseases during adulthood (Joss-Moore et al., 2011; Nistala et al., 2011; Pinney and Simmons, 2010).

Environmental factors can have long-lasting effects on gene expression and chromatin remodeling. Dynamic interactions between adverse environmental factors and central nervous system underpin the pathogenesis of various neurological disorders (Qureshi and Mehler, 2010). Developing brain in fetus and neonate is especially sensitive to environmental signals. Epigenetics refers to the heritable changes in gene expression without alterations in DNA sequence (Holliday, 1987). Epigenetics reveals molecular and cellular processes responsible for modification of gene expression and functional gene networks, mainly consisted of DNA methylation; histone code modifications, nucleosome remodeling, and higher-order chromatin formation; noncoding RNA; and RNA editing, etc., which occurs in epigenome throughout life and controls normal development, adult homeostasis, aging and responses to environmental stimuli, implicating in orchestrating a seemingly infinite of molecular and cellular processes essential for higher nervous system functions and evolution innovations (Mehler, 2008; Qureshi and Mehler, 2010). Recent studies have revealed the key roles of epigenetic

mechanisms in the susceptibility and pathogenesis to some complex diseases, such as cancers and neurological diseases (Mehler, 2008).

Emerging evidence from both human studies and animal experiments indicates epigenetic mechanisms deeply implicated in various neurodevelopmental, neurodegenerative and neuropsychiatric diseases, including acute stroke (Mehler, 2008; Qureshi and Mehler, 2010). Epigenetic mechanisms regulate development, homeostasis and plasticity in the CNS which are very sensitive to local and global environmental, vascular, systemic and intrinsic CNS factors. In the brain, epigenetic mechanisms are critical for brain patterning, neural stem cell maintenance, neurogenesis and gliogenesis, synaptic and neural network plasticity and also involved in processing of complicated cognitive functions (learning and memory) (Qureshi and Mehler, 2010). It is not surprising that epigenetics is involved in the molecular and cellular processes underlying various neurological pathogenesis and functional recovery.

DNA methylation is the most well characterized epigenetic mechanism which plays key roles in regulation of gene expression and cellular processes, including genomic stability, X chromosome inactivation and genomic imprinting (Robertson, 2005). Mechanistically, DNA methylation inhibits the process of transcription and promotes binding of methyl-CpG-binding domain proteins (MBDs), such as MeCP2, which recruit regulatory complexes containing epigenetic factors to methylated genomic loci in order to coordinately orchestrate reversible as well as long-term gene silencing events. Studies have revealed that MeCP2 is essential for progressive stages of postnatal brain development and mature neuronal function, modulates synaptic function and plasticity, autonomic responses and sophisticated neurobehavioral functions, regulates

axonal pathfinding and neural network development, preferentially involved in a spectrum of neurodevelopmental and neuropsychiatric conditions (Mehler, 2008). In general, DNA methylation occurs at CpG rich regions (CpG islands); emerging evidence indicates that site-specific CpG methylation at promoter region plays critical roles in gene transcription and implicates in various physiological and pathological conditions. DNA methyltransferases (DNMTs) mediate DNA methylation by transferring methyl groups from *S*-adenosyl-methionine to cytosine residues in various genomic regions. Members of this enzymatic family include DNMT3a and DNMT3b, which stimulate de novo methylation involved in dynamic DNA methylation, and DNMT1, which presents high levels in the embryonic nervous system and actively maintains the methylation profiles. The expression levels and functions of these factors in neural cells are exquisitely regulated in an activity-dependent manner throughout development and adult life and are responsible for modulating neural subtype specification, maturation, and survival (Sharma et al., 2008; Qureshi and Mehler, 2010).

Aberrant DNA methylation has been linked to a broad spectrum of diseases, such as stroke, atherosclerosis, obesity, diabetes, kidney disease, cancer and autoimmunitic disorders (Robertson, 2005; Gluckman et al., 2009). The most intensely studied neurological diseases correlated with epigenetic changes is Rett syndrome; patients with Rett syndrome present neurodevelopmental defects associated with mutations in the X-linked gene *MECP2*, which encodes the methyl CpG binding protein 2, that binds to methylated DNA (Amir et al., 1999; Esteller, 2006). Other mental retardation disorders are also linked to the disruption of genes involved in epigenetic mechanisms, such as alpha thalassaemia/mental retardation X-linked syndrome, Rubinstein-Taybi syndrome,

and Coffin-Lowry syndrome (Urdinguio et al., 2009). Moreover, aberrant DNA methylation and histone modifications, at a genome-wide level, have recently been considered to be linked to common neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease, and in other neurological disorders such as multiple sclerosis, epilepsy, and amyotrophic lateral sclerosis (Urdinguio et al., 2009).

Of importance, epigenetic mechanisms are also described in cerebral ischemia insults (Qureshi and Mehler, 2010). DNA methylation–mediated regulation of specific genes also plays a role in the pathophysiology of stroke. DNA methylation levels are increased in ischemic brain tissue following middle cerebral artery occlusion (MCAO) in mice and may be responsible for increased cell death (Endres et al., 2000, 2001). Besides, DNA methylation inhibitor administration can reduce ischemic injury after MCAO. Transgenic mice with lower level of DNMT1 demonstrate smaller infarct size following MCAO (Endres et al., 2000, 2001). DNA methylation may make multifaceted effects in cerebral ischemia and influences the susceptibility of CNS to various insults (Qureshi and Mehler, 2010). All of these findings indicate that dynamic modulation of DNMT expression and DNA methylation status represents an important mechanism in regulation of cell death or survival in cerebral ischemia, offering us a promising interventional target. Interestingly, recent studies indicate that differential DNA methylation profiles mediate sex differences in the endogenous neuroprotective response to MCAO. Dramatic upregulation of the estrogen receptor α (ERα) is an endogenous response to MCAO that attenuates ischemic cell death in young female rats (Wilson and Westberry, 2009). Although ERα is primarily expressed during neonatal development, MCAO induces

selective demethylation of the ERα gene promoter in females, leading to the increase in ERα expression and confers additional protective effects in response to ischemic insults (Westberry et al., 2008).

Histone code modification represents another important epigenetic process which mainly functions at single nucleotide and accounts for modulating chromatin structure. The histone code refers to profiles of posttranslational modifications of histone proteins (e.g., acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and adenosine diphosphate– ribosylation) that are catalyzed by specific enzymes (e.g., histone acetyltransferases and histone deacetylases [HDACs]) (Jenuwein and Allis, 2001). It is increasingly recognized that dynamic modulation of chromatin states is associated with the molecular mechanisms that mediate neural cell death and protective responses in stroke (including excitotoxicity, oxidative stress, inflammation, cell cycle regulation, DNA repair, and apoptosis). Abnormal chromatin may be a key feature of necrotic cell death and apoptotic cell death, which are both closely associated with neural injury in stroke. Although the different roles played by chromatin regulation in the pathophysiology of cerebral ischemia are not well characterized, emerging evidence suggests that these functions are extremely important and, furthermore, that chromatin-modifying agents may be neuroprotective (Langley et al., 2009). Study has showed that brain tissue subjected to MCAO exhibits significant changes in histone acetylation (Faraco et al., 2006). Several preclinical studies have also shown that the administration of various HDAC inhibitors in animal models of cerebral ischemia decreases the extent of neuronal injury and improves functional outcomes levels (Faraco et al., 2006). In addition, histone acetylation also plays a role in the protection of neurons against

oxidative stress by indirectly promoting the function of neuroprotective antioxidant enzymes (i.e., peroxiredoxins) (Soriano et al., 2009). A recent review has also suggested that these agents are generally effective in promoting neuroprotection (Langley et al., 2009).

MicroRNAs represent one of the best-characterized subclass of ncRNAs up to now. MicroRNAs regulate developmental and homeostatic gene expression programs in a highly environmentally responsive manner and are implicated in neural differentiation, maintenance, and plasticity (Schratt, 2009). Cerebral ischemia in animal models is associated with highly selective and temporally regulated profiles of miRNAs in the postischemic brain (Jeyaseelan et al, 2008; Dharap et al., 2009). Differential expression patterns of miRNAs in the postischemic brain correlates with differential expression of their target mRNAs, many of which may be implicated in transcriptional regulation, ionic flux, inflammation, and other stress responses. These results suggest that miRNA networks may regulate a spectrum of pathological processes in the postischemic brain. For example, MicroRNA-140 is one of the miRNAs that was rapidly upregulated in the brain 3 hours after middle cerebral artery occlusion and sustained for 72 hours. One of the validated target mRNAs for miRNA-140 encodes stromal cell– derived factor 1, which plays an important role in the CNS by mediating neural progenitor cell proliferation and migration and tissue repair after cerebral ischemia (Nicolas et al., 2008), suggesting miRNA-140, at least in part, implicated in rehabilitating response after brain ischemic insult. Another neurological disease most frequently associated with miRNAs is fragile X syndrome, which manifests with various severity of a range of cognitive or intellectual disabilities and caused by mutations in the *FMR1* gene located on the long

arm of the X chromosome. *FMR1* encodes FMRP, which is an RNA binding protein and associated with RISC and with miRNAs themselves (Jin et al., 1998; Kosik, 2006).

A number of epigenetic agents, such as DNA methylation and HDAC inhibitors, have already been evaluated in preclinical and clinical trials (Mehler, 2008). However, these therapeutic approaches are still in their infancy. Existing agents usually exert relatively nonspecific effects directed towards a restricted number of epigenetic processes. Importantly, it provides us with another promising interventional target to overcome those complex neurological diseases.

Central Hypothesis

The central hypothesis of our project is that perinatal nicotine exposure causes epigenetic programming of aberrant expression patterns of AT_1R and/or AT_2R in the developing brain, resulting in heightened susceptibility to neonatal hypoxic ischemic brain injury.

Significance

Both human and animal studies have supported the notion of developmental origins of adult health and disease. Prenatal stress including nicotine exposure exerts great impacts on the fetus during the vulnerable developmental stage at multifaceted levels, which may contribute to adverse programming of ischemic-sensitive phenotype in the developing brain and heightened vulnerability of neonatal hypoxic-ischemic encephalopathy and long-term neurodevelopmental disorders. Cigarette smoking represents the single largest modifiable neuropharmacological exposure for the fetus and

newborn (Wickstrom, 2007). Nowadays, neonatal hypoxic-ischemic encephalopathy (HIE) is still one of major causes of acute mortality as well as chronic neurological disability in newborns. Because of current deficiency in potent and effective therapy, the prognosis and outcome for most neonatal HIE are less than optimal at the best, which makes urgently necessary for further exploration and investigation of pathophysiology and underlying mechanisms for the heightened neonatal HIE. Epigenetic modification of gene expression patterns provides us with a promising interventional target although it is still in its infancy stage. Further studies on the epigenetic regulation of AT_1R and AT_2R gene expression patterns induced by perinatal nicotine exposure in the developing brain should provide more insights into mechanisms at the molecular level and may suggest new insights of preventable and therapeutic strategies that may finally contribute to the treatment of HIE in newborns.

CHAPTER TWO

PERINATAL NICOTINE EXPOSURE INCREASES VULNERABILITY OF HYPOXIC-ISCHEMIC BRAIN INJURY IN NEONATAL RATS: ROLE OF ANGIOTENSIN II RECEPTORS

By

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Abstract

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 (AT_1R) and type 2 (AT_2R) receptors in the developing brain. Nicotine was administered to pregnant rats through subcutaneous osmotic minipumps. HI brain injury was determined in 10-day-old pups. AT_1R and AT_2R expression patterns were assessed through Western blotting, quantitative polymerase chain reaction, immunofluorescence, and confocal imaging. 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 3 bases upstream of TATA-box at the 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. Perinatal nicotine exposure causes epigenetic repression of the AT_2R gene in the developing brain resulting in heightened brain vulnerability to HI injury in neonatal male rats in a sex-dependent manner.

Introduction

Hypoxic–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 (Ferrieo, 2004; Verklan, 2009). 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 (Jensen, 2006). 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 (Lambers and Clark, 1996).

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 (Wickstrom, 2007; Pauly and Slotkin, 2008). 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 (AT_1R) and type 2 (AT_2R) receptors are pivotal players in the pathogenesis of ischemic brain injury (Sokol et al., 2004; Schrader et al., 2005; Ando et al., 2004), we sought to investigate further the role of AT_1R and AT_2R in the nicotine-

mediated ischemia-sensitive phenotype of neo-natal brains. 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 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 (Lawrence et al., 2011). 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 Injection

A modified Rice-Vannucci model was conducted in P10 pups (Vannucci, et al., 1999). 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, before 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/min with a 10 μL syringe (Stoelting) 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 (Han and Holtzman, 2000). 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 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 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 AT_1R (1:100) and AT_2R (1:1000; Santa Cruz Biotechnology; Santa Cruz, CA) as described previously (Xue et al., 2011). After washing, membranes were incubated with secondary horseradish peroxidaseconjugated 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 $AT_{1a}R$, $AT_{1b}R$, and AT_2R mRNA was determined by real-time reverse transcription– polymerase chain reaction using an Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously (Xue et al., 2011). 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’-tgacctcccatctcctttg-3’ (reverse); and AT_2R, 5’- caatctggctgtggctgactt-3’ (forward) and 5’-tgcacatcacaggtccaaaga-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 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 μmol/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 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_2R gene promoter was determined as previously described (Lawrence et al., 2011; Pattersen et al., 2010). 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 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 polymerase chain reaction at CpG_{-52} locus (forward primer, 5'-ttttttggaaagttggtaagtgttta-3'; reverse primer for C, 5'-ctctaatttccttcttatatattca-3'; reverse primer for Cm, 5'- ctctaatttccttcttatatattcg-3') and

CpG_{+11} locus (forward primer, 5'- gaaggttttttagtggatag- 3'; reverse primer for C, 5'-aaaaaaaactttcaattctatactca- 3'; reverse primer for C^m, 5'-aaaaaaaactttcaattctatactcg-3'), respectively. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference gene. Real-time methylation-specific polymerase chain reaction was performed using the iQ SYBR Green Supermix with iCycler real-time polymerase chain reaction 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 (Lawrence et al., 2011; Pattersen et al., 2010).

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) after heat-induced procedures. The following primary antibodies were used: mouse antineuronal nuclei (Millipore); mouse anti glial fibrillary acidic protein (Millipore); rabbit anti-AT_1R (Santa Cruz); and rabbit anti-AT_2R (Santa Cruz). After blocking with 1% bovine 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 (Xiong et al., 2012).

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 3A). In P10 pups, both body and brain weight were decreased but the brain to body weight ratio remained significantly increased in both sexes (Figure 3B), suggesting asymmetrical growth restriction in the fetus and neonate in nicotine-treated animals.

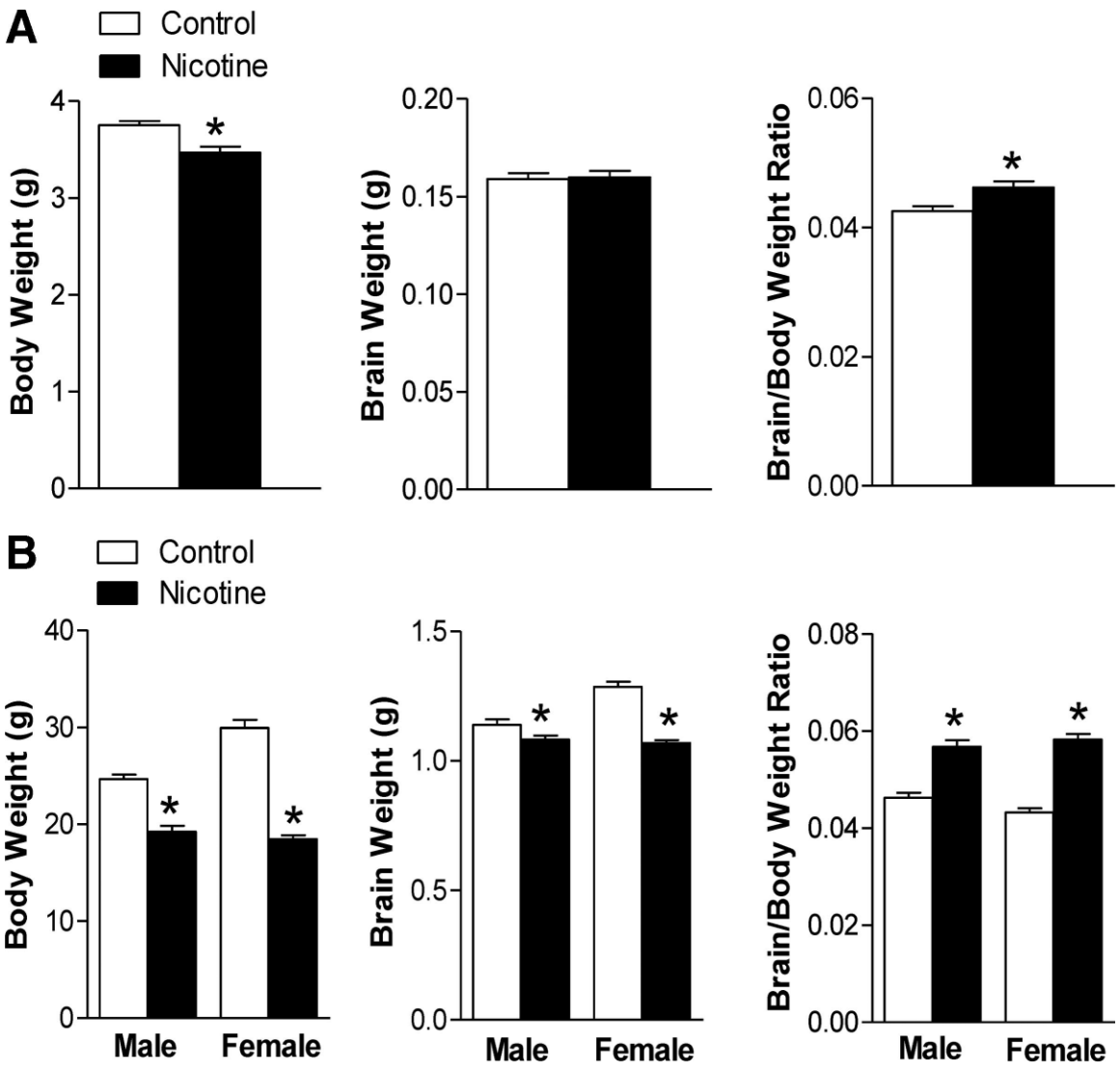

Figure 3. Effect of nicotine on body weight, brain weight, and brain to body weight ratio in E21 fetuses (**A**; n = 27–39) and P10 pups (**B**; n = 9–13). Data are mean ± SEM. **P*<0.05 versus control group. P10 indicates 10-day-old neonatal.

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 4). The nicotine treatment significantly exaggerated HI-induced brain infarct size in male, but not female, pups (Figure 4).

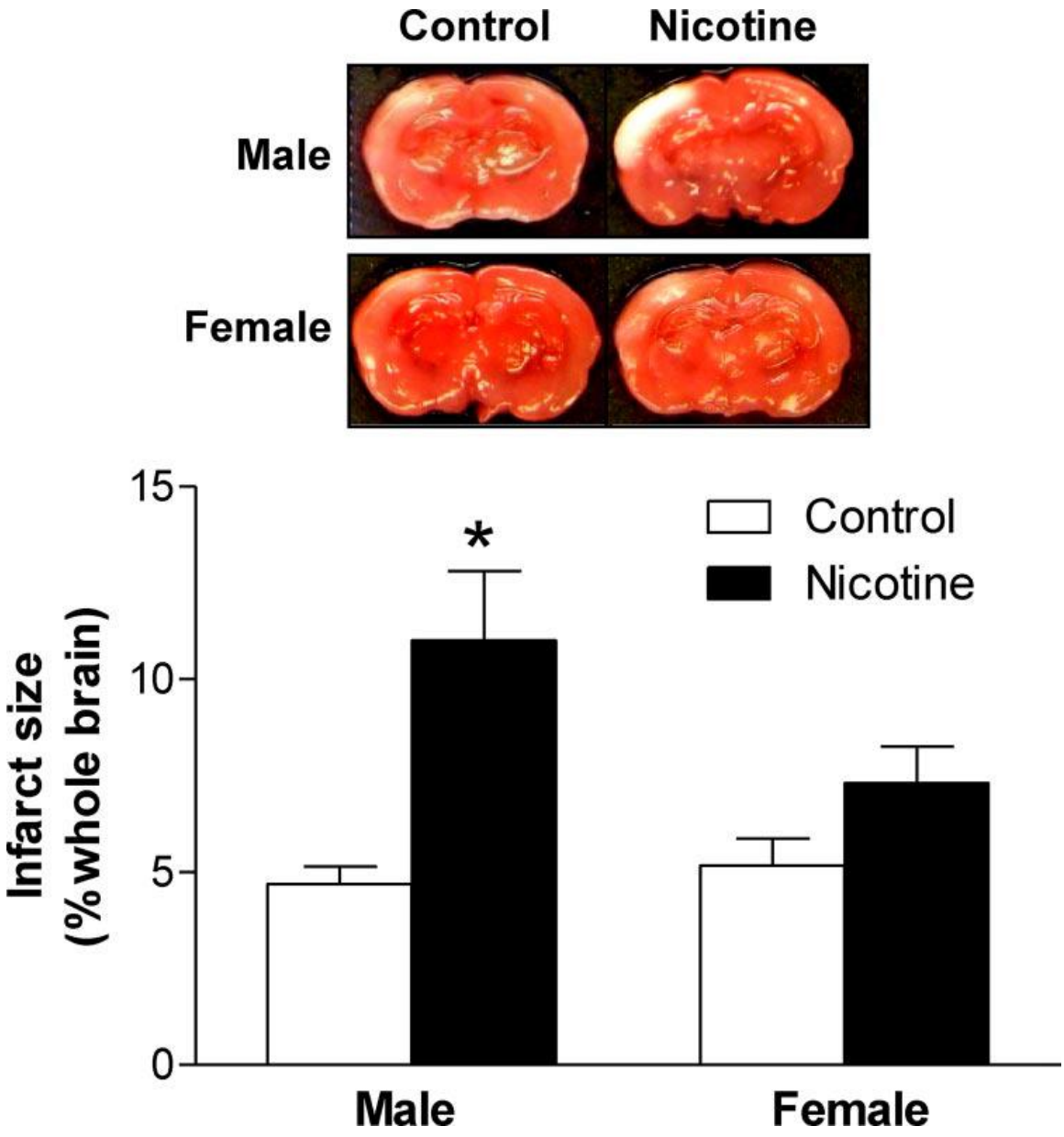

Figure 4. Effect of nicotine on HI-induced brain infarct size in P10 pups. Data are mean ± SEM, n = 4 to 6. *P<0.05 versus control group. HI indicates hypoxia–ischemia; P10, 10-day-old neonatal.

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 5A–B). 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 5B). In P10 pups, brain AT_2R protein and mRNA abundance were significantly decreased in male pups in nicotine-treated animals (Figure 5C–D). In contrast, in female pups, nicotine caused a significant increase in brain AT_2R protein and mRNA abundance (Figure 5C–D). 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 5C–D). However, nicotine induced a significant reduction of brain AT_1R protein and $AT_{1a}R$ mRNA abundance in female pups (Figure 5C–D). Immunofluorescence and confocal imaging analyses showed that both AT_1R and AT_2R presented in neurons but not in astrocytes in the cortex (Figure 6) and hippocampus (Figure 7) of P10 pups. It appeared that nicotine treatment increased astrocyte numbers in both cortex and hippocampus (Figure 8).

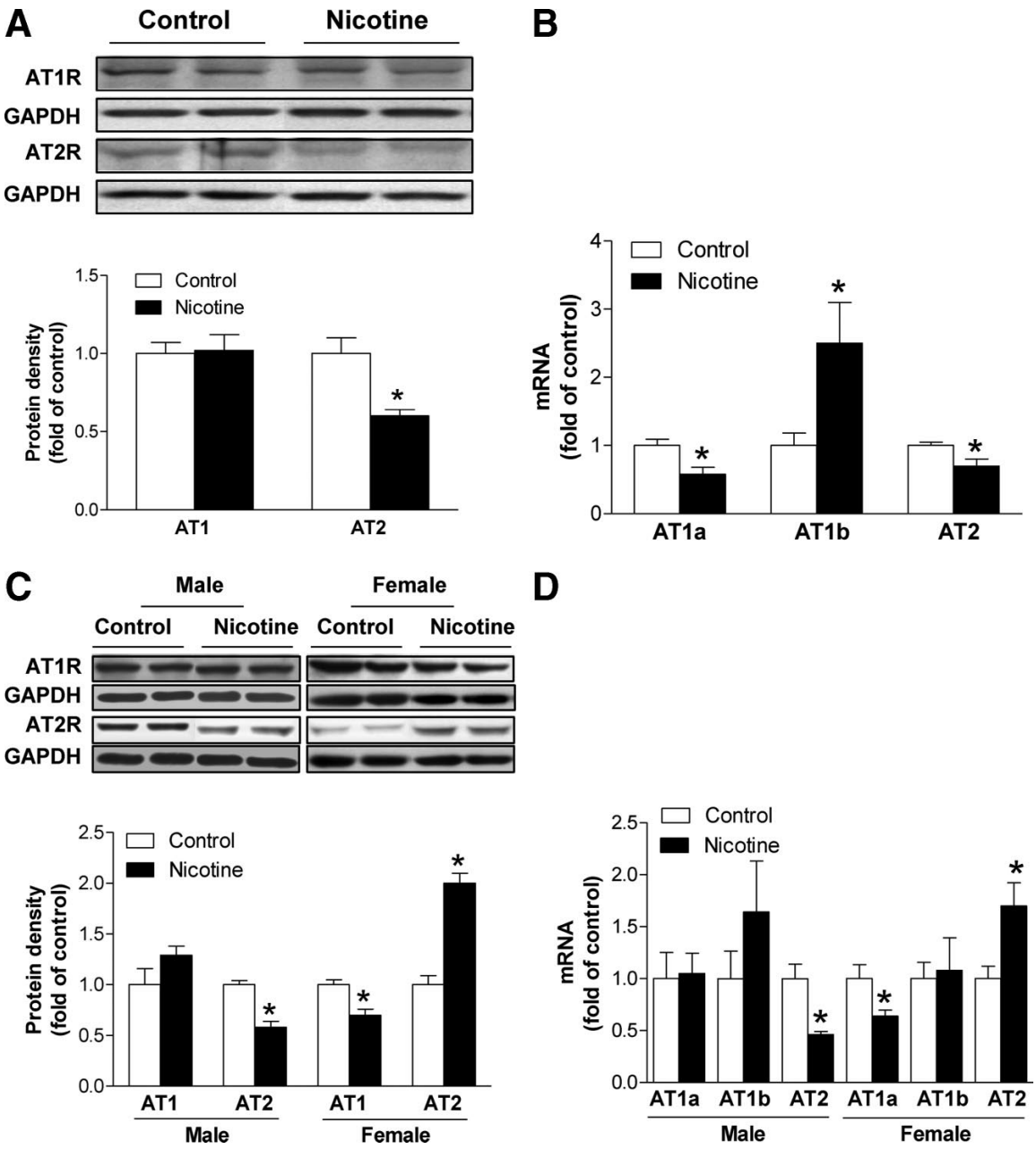

Figure 5. Effect of nicotine on protein and mRNA abundance of AT_1R and AT_2R in E21 fetal (**A**–**B**) and P10 pup (**C**–**D**) brains. Data are mean ± SEM, n = 4 to 6. **P*<0.05 versus control group. AT_1R indicates angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; P10, 10-day-old neonatal.

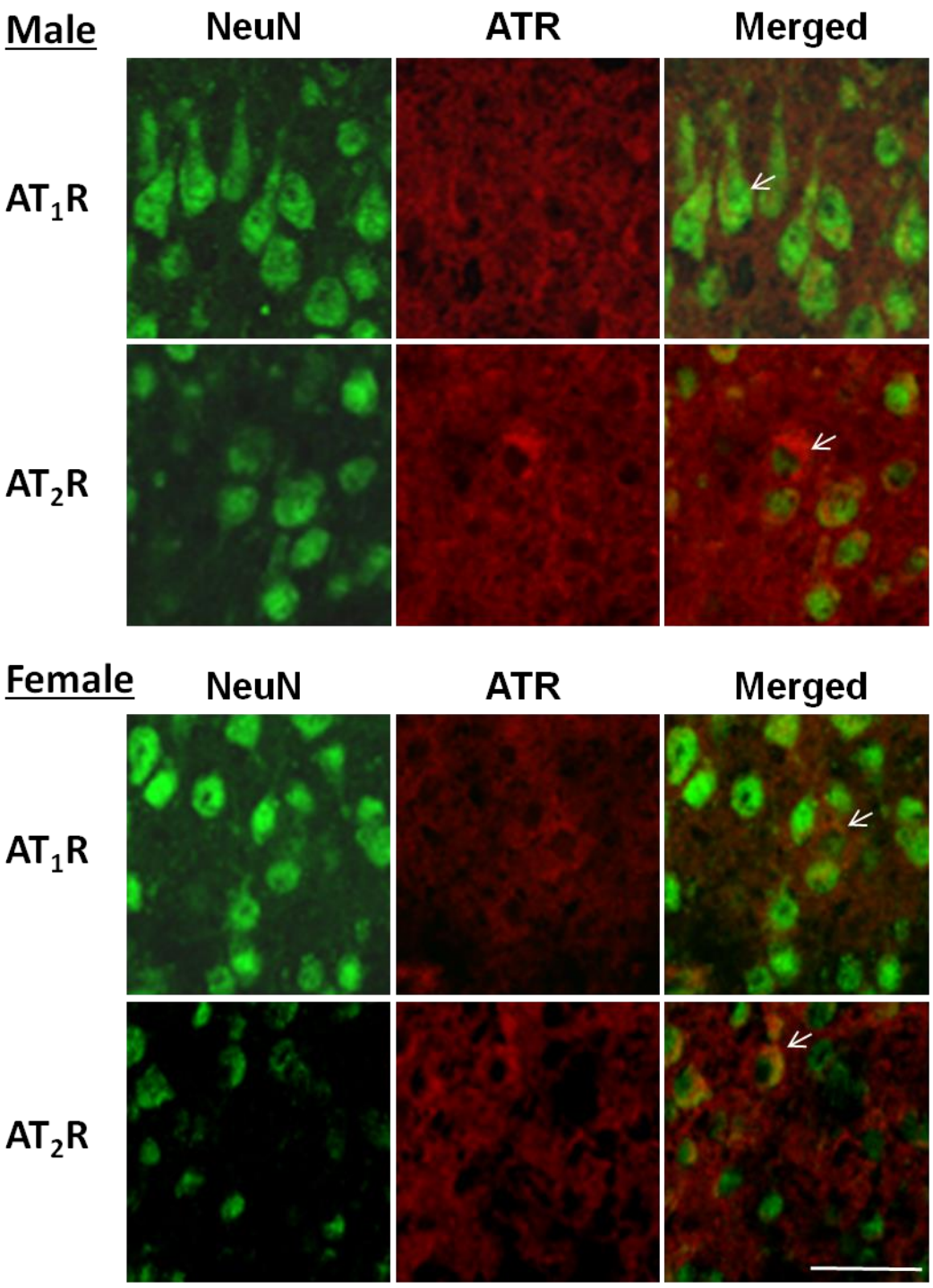

Figure 6. Immunoreactivity of AT_1R and AT_2R in the cortex of P10 pup brains. NeuN is visualized by FITC (green). AT_1R and AT_2R are visualized with Texas Red (red). Arrows show the peri-nuclear location of AT_1R and AT_2R. Scale bar: 50 μm. Representative immunofluorescence confocal images of samples from 3 animals in each group are shown.

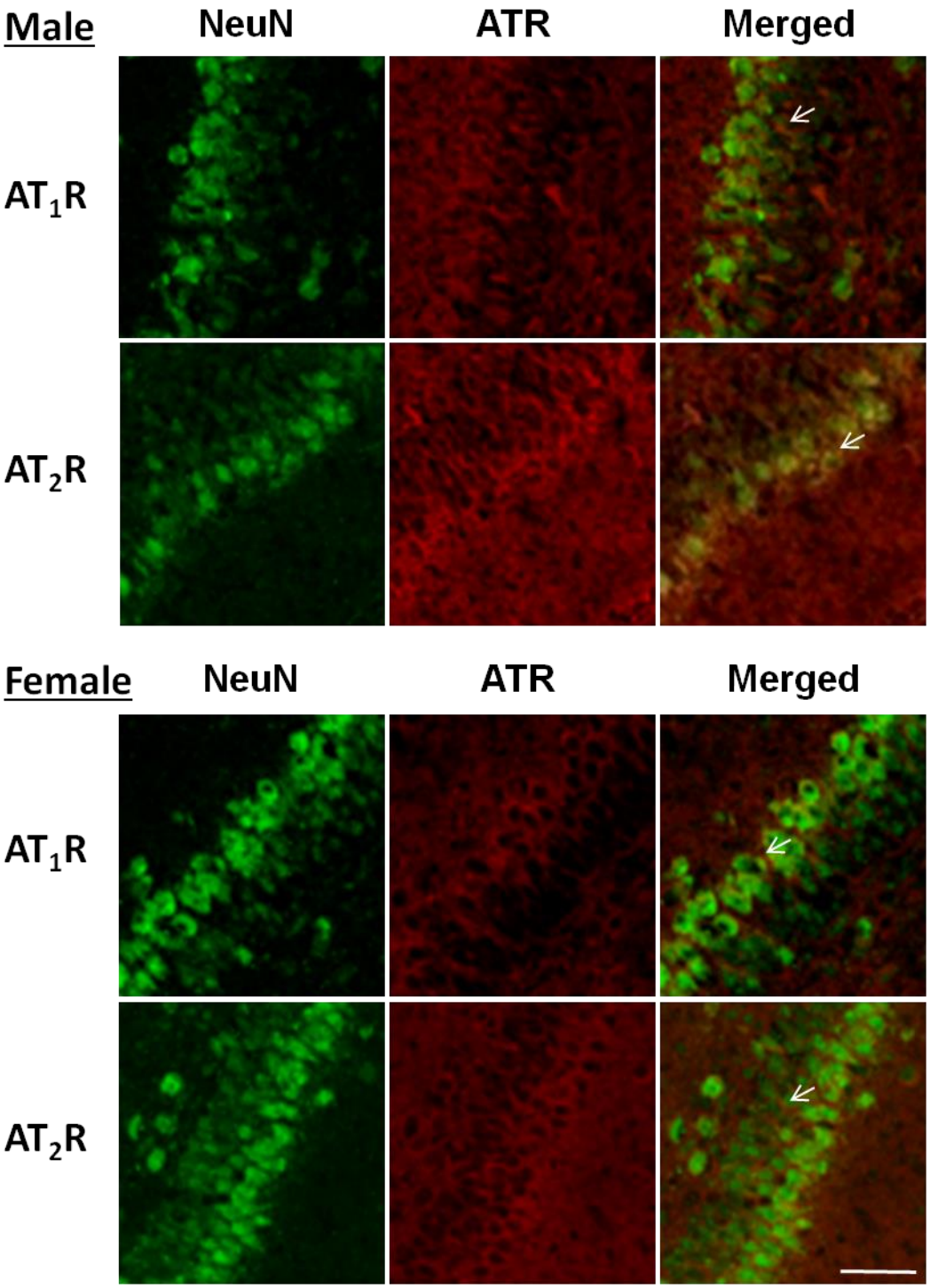

Figure 7. Immunoreactivity of AT_1R and AT_2R in the hippocampus of P10 pup brains. NeuN is visualized by FITC (green). AT_1R and AT_2R are visualized with Texas Red (red). Arrows show the peri-nuclear location of AT_1R and AT_2R. Scale bar: 50 μm. Representative immunofluorescence confocal images of samples from 3 animals in each group are shown.

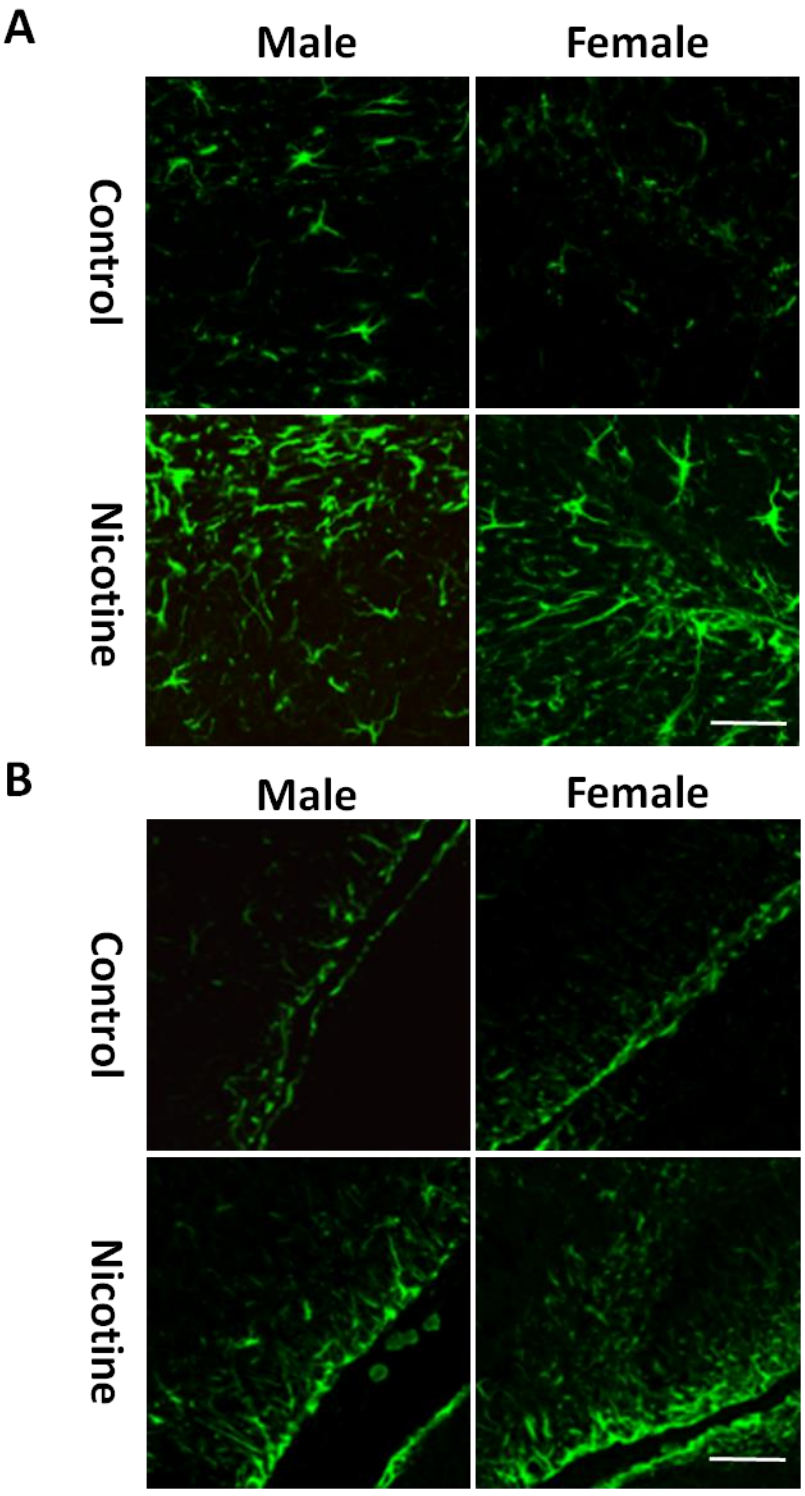

Figure 8. Immunostaining of astrocytes with GFAP in the cortex (**A**) and hippocampus (**B**) of P10 pup brains. Scale bar: 50 μm. Representative immunofluorescence confocal images of samples from 3 animals in each group are shown.

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 first evaluated the role of AT_1R and AT_2R in the pathogenesis of HI brain injury in pups through intracerebroventricular (ICV) injection of AT_1R or AT_2R antagonists. Compared with the saline control, ICV of either losartan (Figure 9A) or PD123319 (Figure 9B) 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.

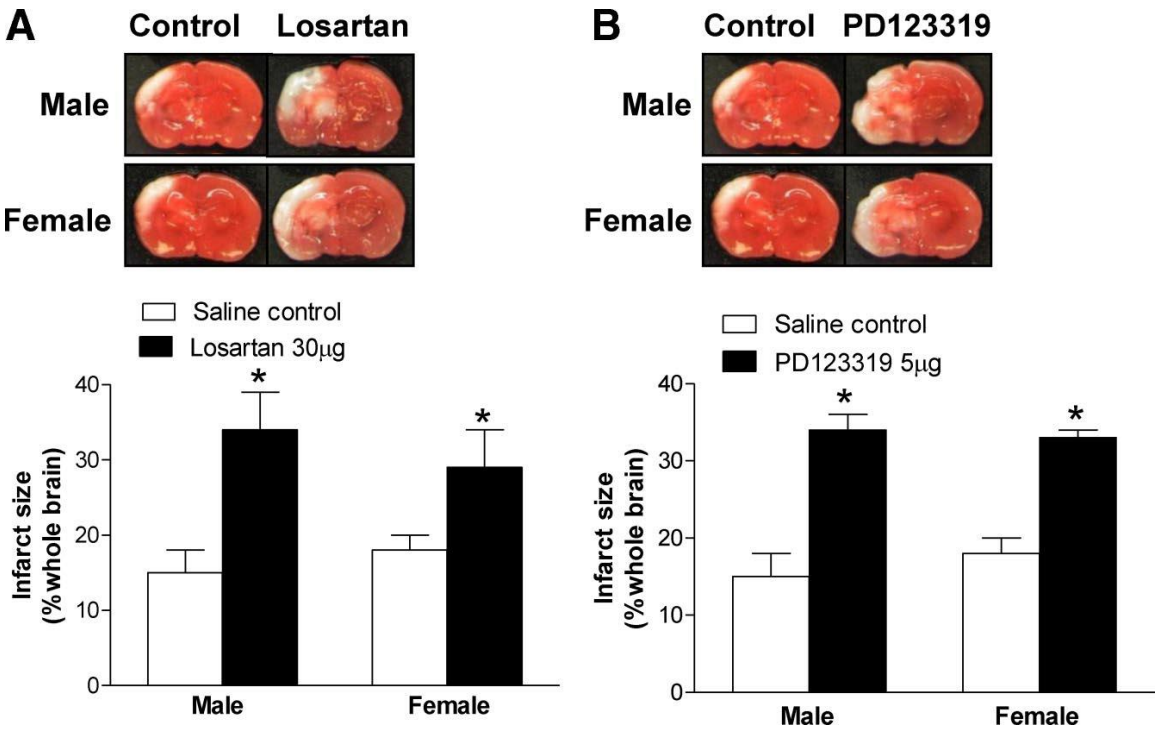

Figure 9. Effect of losartan (**A**) and PD123319 (**B**) on HI-induced brain infarct size in P10 pups. Data are mean ± SEM, n = 4 to 6. **P*<0.05 versus control group. HI indicates hypoxia–ischemia; P10, 10-day-old neonatal.

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 10A, 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 10B, 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 10B).

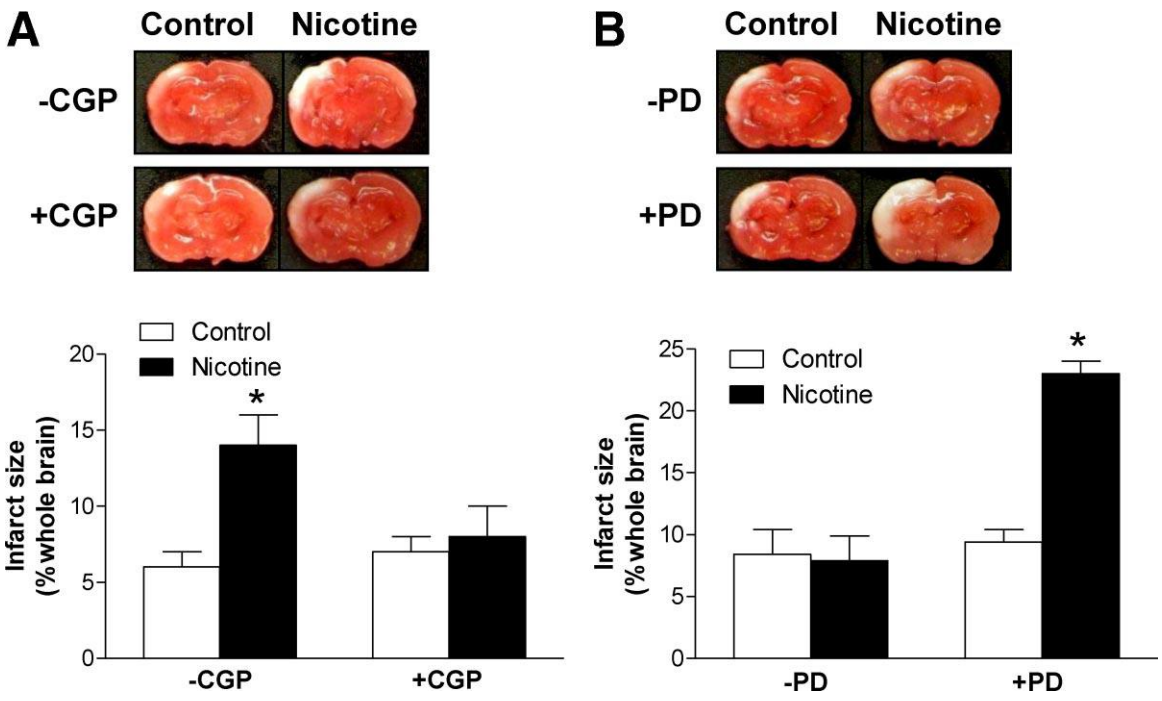

Figure 10. Effect of CGP42112 (CGP) in male P10 pups (**A**) and PD123319 (PD) in female P10 pups (**B**) on nicotine-induced changes in brain HI injury. Data are mean ± SEM, n = 4 to 8. **P*<0.05 versus control group. P10 indicates 10-day-old neonatal; HI, hypoxia–ischemia.

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 (Xue et al., 2011). Two CpG loci were identified at the AT_2R promoter; one was located 3 bases upstream of TATA-box (CpG_{-52}) and the other one 11 bases downstream of transcription start site (CpG_{+11}). 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 (Kitazawa and Kitazawa, 2007). As shown in Figure 11, 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.

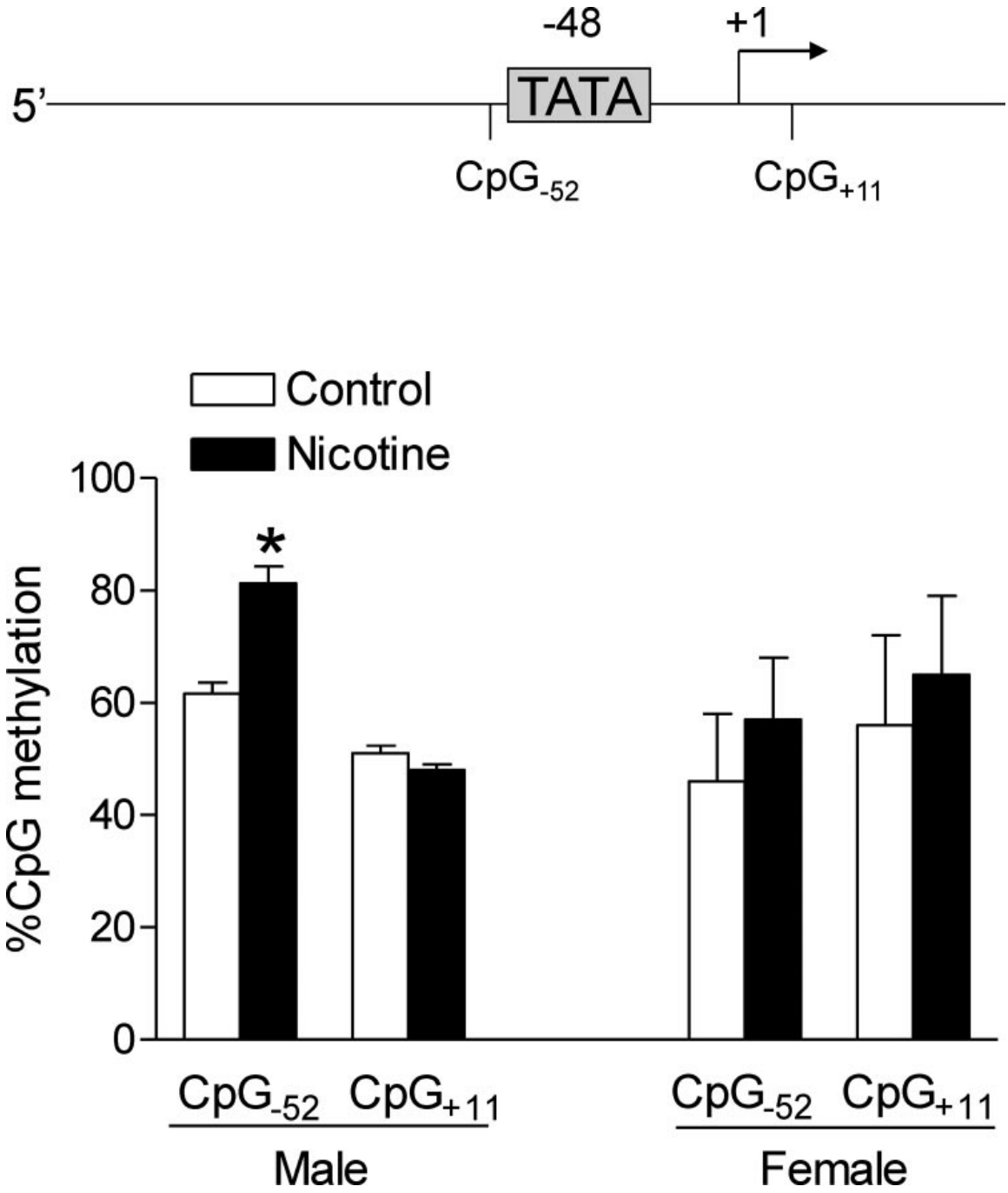

Figure 11. Effect of nicotine on methylation of CpG loci at AT_2R promoter in P10 pup brains. Data are mean ± SEM, n = 5 to 10. *P<0.05 versus control group. AT_2R indicates angiotensin II type 2 receptor; P10, 10-day-old neonatal.

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 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 3 bases upstream of TATA-box at the 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 (Lambers and Clark, 1996; Slotkin, 1998). Nicotine readily crosses the placenta into the fetal circulation, resulting in fetal nicotine concentrations being 15% higher than maternal levels (Koren, 1995). 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 (Yager and Ashwal, 2009). 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 (Arbeille et al., 1992: Onal et al., 2004; Mao et al., 2008), these effects were not observed in continuous low-level infusion of nicotine through a minipump (Slotkin, 1998).

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, whereas fetal brains express high levels of AT_2R that decrease during the postnatal development (Millan et al., 1991). 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 (Li et al., 2005). The neuroprotective effect of AT_2R demonstrated in the present study is consistent with previous findings (Li et al., 2005; Iwai et al., 2004; McCarthy et al., 2009). In contrast, the present finding of a 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 (Ando et al., 2004; Dai et al., 1999; Lou et al., 2004; Zhou et al., 2005). These findings highlight the important differences between immature and mature brains in AT_1Rmediated 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 (Rothman and Olney, 1986; Sidhu et al., 1997). Although long-term and systemic administration of AT_1R antagonists often showed neuroprotective effects of the brain through 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 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 AT_1R antagonists in the setting of acute ischemic injury in the heart (Xue et al., 2011; Ford et al., 1996) 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 the AT_2R agonist CGP42112 abrogated 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 (McCarthy et al., 2009). 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 (Xue et al., 2011). The finding that nicotine treatment significantly increased methylation of CpG_{-52} locus 3 bases upstream of TATA-box at the 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 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 (Kitazawa and Kitazawa, 2007). 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 $PKC\varepsilon$ promoter in the developing heart, which causes $PKC\varepsilon$ gene repression (Lawrence et al., 2011). 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 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 (Patterson et al., 2010). 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 (Xue et al., 2011). Additionally, it has been shown that estrogen receptors mediate the downregulation of AT_1R but upregulation of AT_2R in rodents (Armando et al., 2002; Baiardi et al., 2005; Rodriguez-Perez et al., 2012). 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 (Barker and Osmond, 1986; Bateson et al., 2004; Gluckman et al., 2008; McMillen and Robinson, 2005).

The present investigation provides novel evidence that perinatal nicotine exposure increases brain susceptibility to HI injury through 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. 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.

Acknowledgments

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CHAPTER THREE

PROMOTER METHYLATION SUPRESSES AT_2R GENE AND INCREASES BRAIN HYPOXIC-ISCHEMIC INJURY IN NEONATAL RATS

by

Yong Li, Daliao Xiao, Shumei Yang and Lubo Zhang

Abstract

Perinatal nicotine exposure down-regulated angiotensin II type 2 receptor (AT_2R) 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 AT_2R 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 AT_2R promoter was determined in the brain by quantitative methylation-specific PCR. Nicotine exposure significantly increased methylation of CpG_{-52} near the TATA-box at AT_2R 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 methyl-binding protein (MeCP2) and a decrease in TBP binding to AT_2R promoter *in vivo* in neonatal brains of nicotine-treated animals. This resulted in AT_2R gene repression in the brain. Intracerebroventricular administration of 5-aza-2'-deoxycytidine abrogated the enhanced methylation of CpG_{-52}, rescued the TBP binding, and restored AT_2R expression. Of importance, 5-aza-2'-deoxycytidine reversed the nicotine-increased vulnerability of brain hypoxic-ischemic injury in the pups. The finding provides mechanistic evidence of increased promoter methylation in the developing brain linking perinatal stress and a pathophysiological consequence of heightened vulnerability of hypoxic-ischemic encephalopathy in neonatal brains.

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 (AT_2R) 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 AT_2R 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 AT_2R 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 AT_2R 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 AT_2R gene promoter, resulting in a decrease in the binding of TATA-binding protein (TBP) to AT_2R promoter and a repression of AT_2R 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 AT_2R 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 (4ug/kg/min) implanted subcutaneously from Day 4 of gestation to Day 10 after birth. All rats were allowed to give birth and further studies were conducted in male 10-day-old neonatal (P10) pups. Female neonatal pups were euthanized on postnatal day 12. 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

As previously reported (Li et al., 2012), a modified Rice-Vannucci model was conducted in male P10 pups (Vannucci et al., 1999). 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 hours. 5-Aza (1mg/kg; Sigma-Aldrich), a DNA methylase inhibitor, was administered intracerebroventricularly on postnatal day 7 (P7), one dosage 3 days before HIE treatment. Briefly, 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. 5-Aza was injected at a rate of 1μL/min with a 10μL syringe (Stoelting) 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 (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. The same procedures were treated in the vehicle group.

Measurement of Infarct Size

As previously described (Li et al., 2012), 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 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 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 120 minutes. Proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies against AT_2R (1:1000; Santa Cruz Biotechnology; Santa Cruz, CA) as described previously (Xue et al., 2011). After washing, membranes were incubated with secondary horseradish peroxidaseconjugated 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 AT_2R mRNA was determined by real-time reverse transcription-polymerase chain reaction using an Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously (Xue et al., 2011). The AT_2R primers used were: 5'-caatctggctgtggctgactt-3' (forward) and 5'-tgcacatcacaggtccaaaga-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 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 μmol/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 Polymerase Chain Reaction (MSP)

CpG methylation at rat AT_2R gene promoter was determined as previously described (Lawrence et al., 2011; Patterson et al., 2010). 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 by a Wizard DNA clean up system (Promega) and resuspended in 40 μL H_2O. Bisulfite-treated DNA was used as a template for real-time fluorogenic methylation-specific polymerase chain reaction at CpG_{-52} locus (forward primer, 5'-ttttttggaaagttggtaagtgttta-3'; reverse primer for C, 5'-ctctaatttccttcttatatattca-3'; reverse primer for Cm, 5'-ctctaatttccttcttatatattcg-3'). Real-time methylation-specific polymerase chain reaction was performed using the iQ SYBR Green Supermix with iCycler real-time polymerase chain reaction 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 (Lawrence et al., 2011; Patterson et al., 2010).

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were collected from brains using NXTRACT CelLytic Nuclear Extraction Kit (Sigma). The oligonucleotide probes with CpG_{-52} and $^{m}CpG_{-52}$ of the TBP binding site at rat AT_2R promoter region were labeled and subjected to gel shift assays using the Biotin 3'end labeling kit and Light-Shift Chemiluminescent EMSA Kit (Pierce Biotechnology, Rockford, IL), as previously described(Lawrence et al., 2011; Patterson et al., 2010). Briefly, single stranded oligos were incubated with Terminal

Deoxynucleotidyl Transferase (TdT) and Biotin- 11-dUTP in binding mixture for 30 min at 37ºC. 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 95ºC 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 µL containing 50 fmol oligonucleotide probes, 1× binding buffer, 1 µg of poly (dI-dC), and 10 µg of nuclear extracts. For competitions studies, increasing concentrations of non-labeled oligonucleotides were added to binding reactions. For super-shift assay, 2 µL 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/cm^2). Membranes were blocked and then visualized using the reagents provided in the LightShift kit.

Chromatin Immunoprecipitation Assay (ChIP)

Chromatin extracts were prepared from brains of male P10 rat pups. ChIP assays were performed using the ChIP-IT kit (Active Motif), as previously described (Patterson et al., 2010; Meyer et al., 2009). 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'-tctggaaagctggcaagtgt- 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 ± 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 AT_2R promoter region resulted in a significant decrease in AT_2R promoter activity (Xue et al., 2011). To demonstrate the binding of TATA-binding protein (TBP) to the TATA element at AT_2R 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 12, lane 2), which was blocked by 200-fold excess of unlabeled oligonucleotide probes in cold competition (Figure 12, lane 4). Super-shift analysis showed that a TBP antibody caused super-shifting of the DNA-protein complex (Figure 12, lane 3). A single CpG_{-52} locus 3 bases upstream of the TATA-box was identified at rat AT_2R 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 13, methylation of CpG_{-52} locus resulted in a significant decrease in the TBP binding affinity to the TATA element.

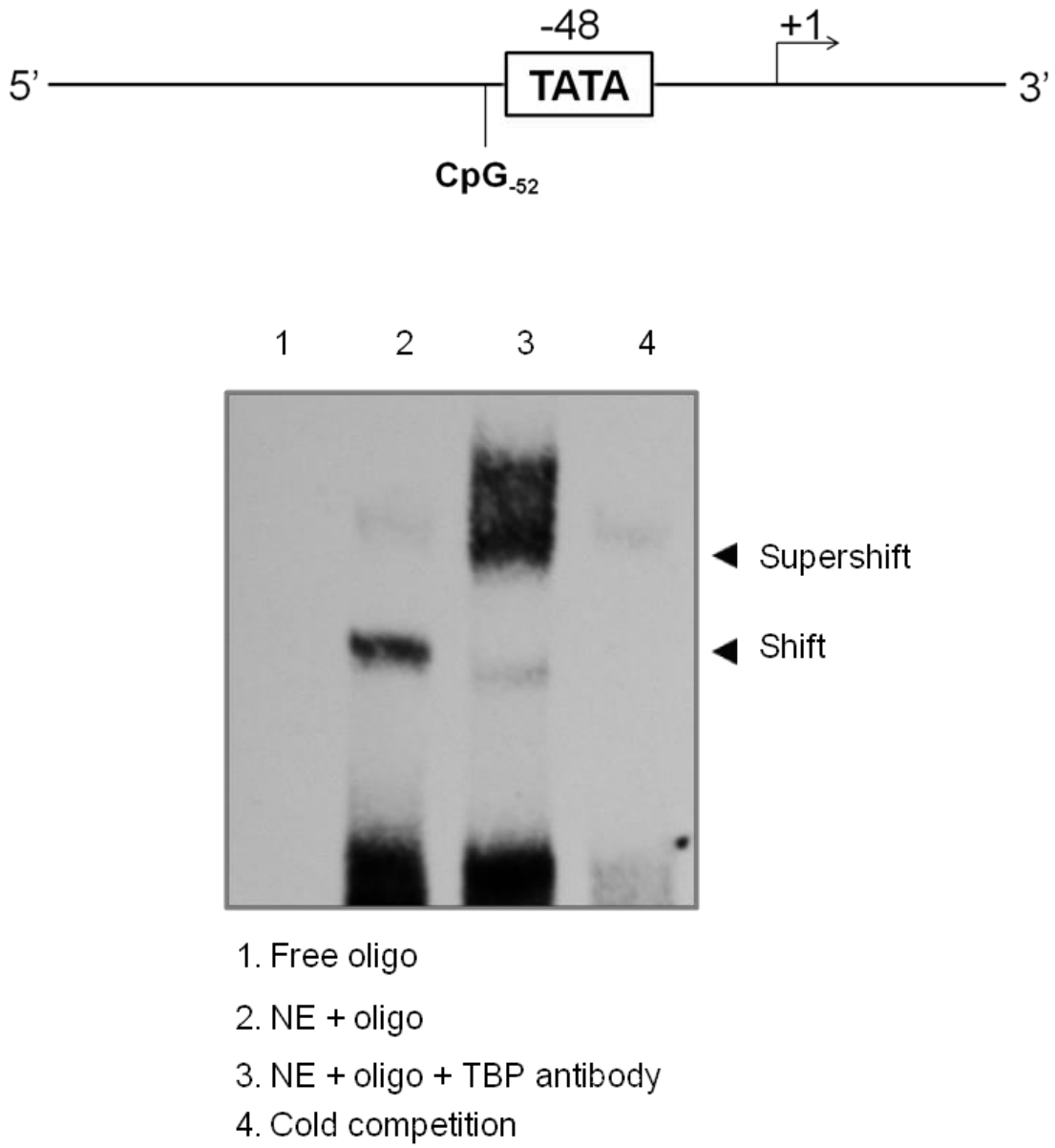

Figure 12. Binding of TBP to TATA element at AT_2R promoter in rat pup brains. Nuclear extracts (NE) from pup brains were incubated with double-stranded oligonucleotide probes containing the TATA element at -48 in the absence or presence of a TBP antibody. Cold competition was performed with unlabeled competitor oligonucleotide at a 200-fold molar excess.

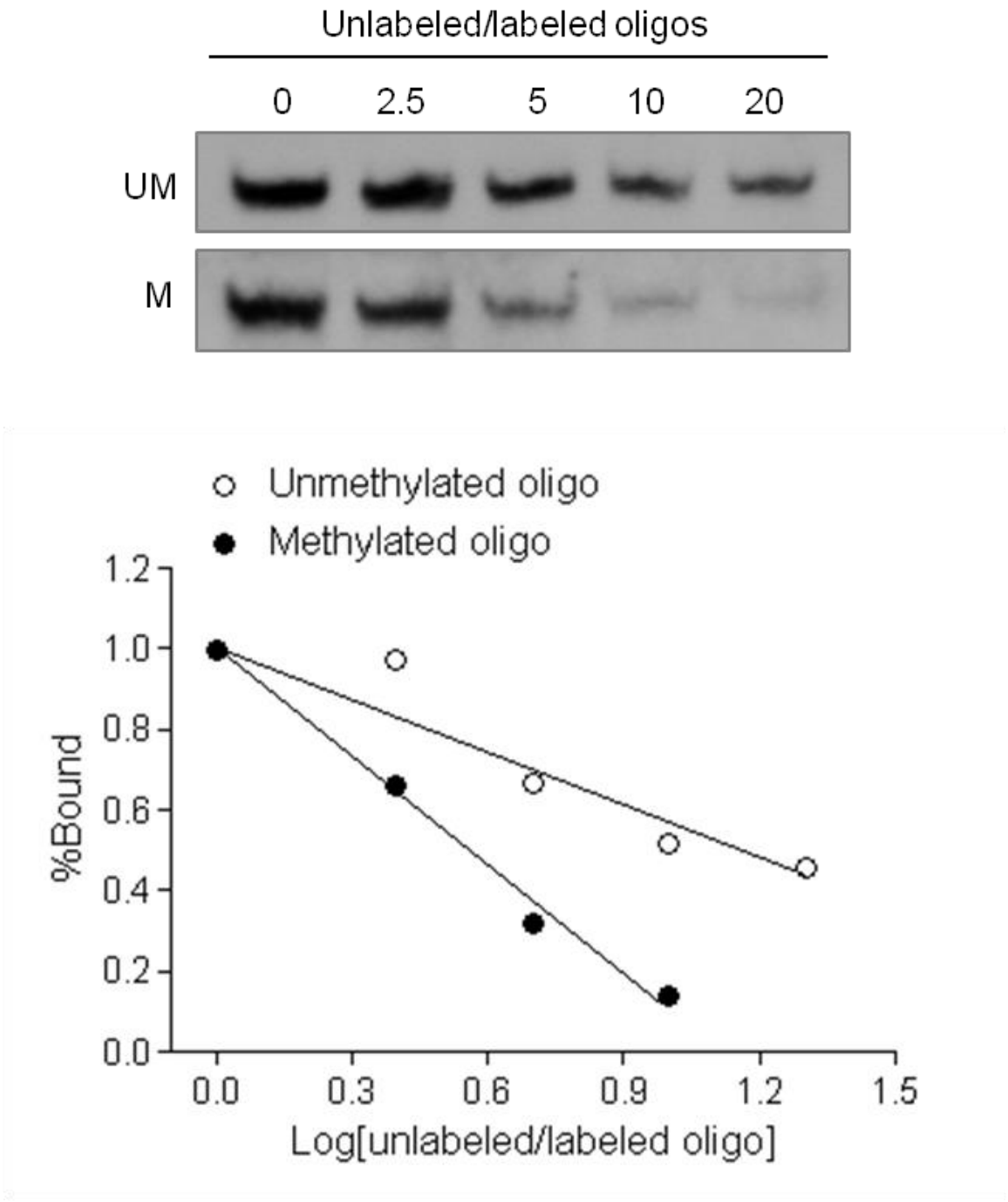

Figure 13. CpG_{-52} methylation inhibited TBP binding affinity at AT_2R promoter. The binding affinity of TBP to the TATA element was determined in competition studies performed in pooled nuclear extracts from pup brains with the increasing ratio of unlabelled/labelled oligonucleotides encompassing the TATA element at -48 with unmethylated (UM) or methylated (M) CpG_{-52} locus.

5-Aza-2'-deoxycytidine Abrogated Nicotine-Induced Methylation of CpG_{-52} Locus and Restored AT_2R Expression

The previous study demonstrated that perinatal nicotine exposure resulted in a down-regulation of AT_2R 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 AT_2R in pup brains, we measured methylation status of the CpG_{-52} locus at AT_2R promoter in male pups in the control and nicotine-treated animals. As shown in Figure 14, 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'-deoxycytodine abrogated the nicotine-induced methylation (Figure 14). We further investigated the functional significance of the nicotine-mediated methylation in regulating TBP binding to AT_2R promoter *in vivo* in the context of intact chromatin *via* a ChIP approach. As shown in Figure 15, 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 AT_2R promoter in pup brains. 5-aza-2'-deoxycytodine blocked these nicotine-induced effects (Figure 15). Consistently, 5-aza-2'-deoxycytodine restored the nicotine-induced down-regulation of AT_2R mRNA and protein expression in the brains (Figure 16).

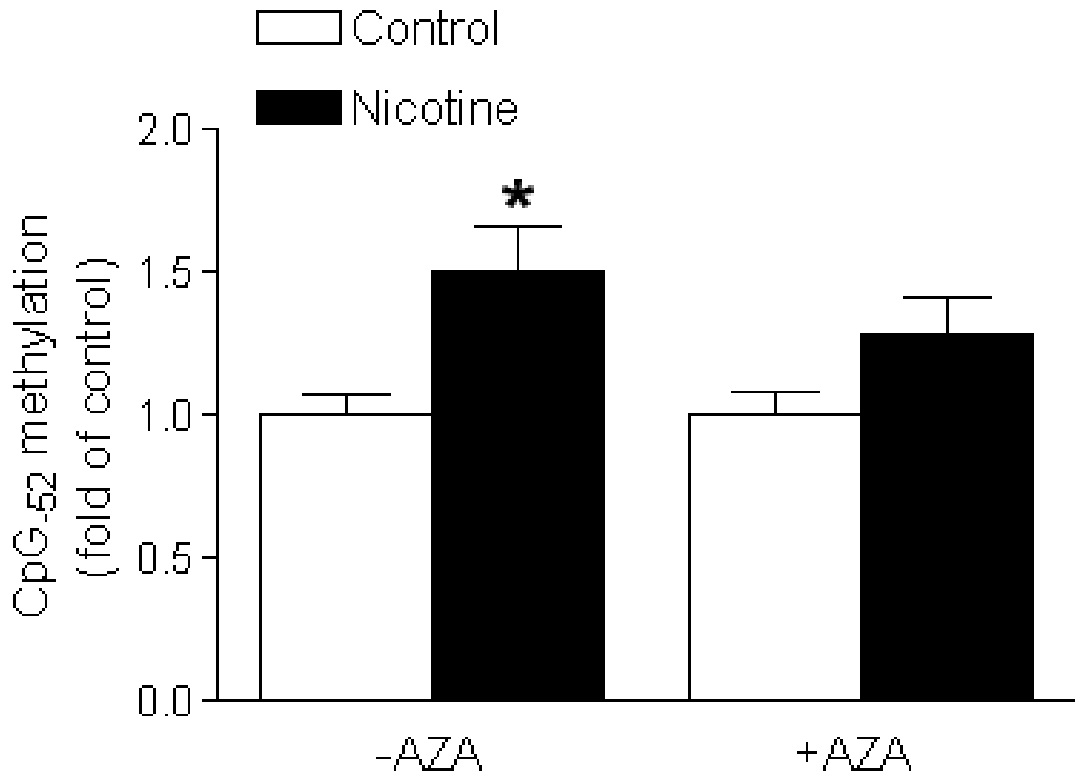

Figure 14. **5**-Aza-2'-deoxycytidine abrogated nicotine-induced methylation of CpG_{-52} locus. Methylation of CpG_{-52} locus at AT_2R promoter was determined in pup brains isolated from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA). Data are means ± SEM, $n = 5$. *$P < 0.05$ versus control group.

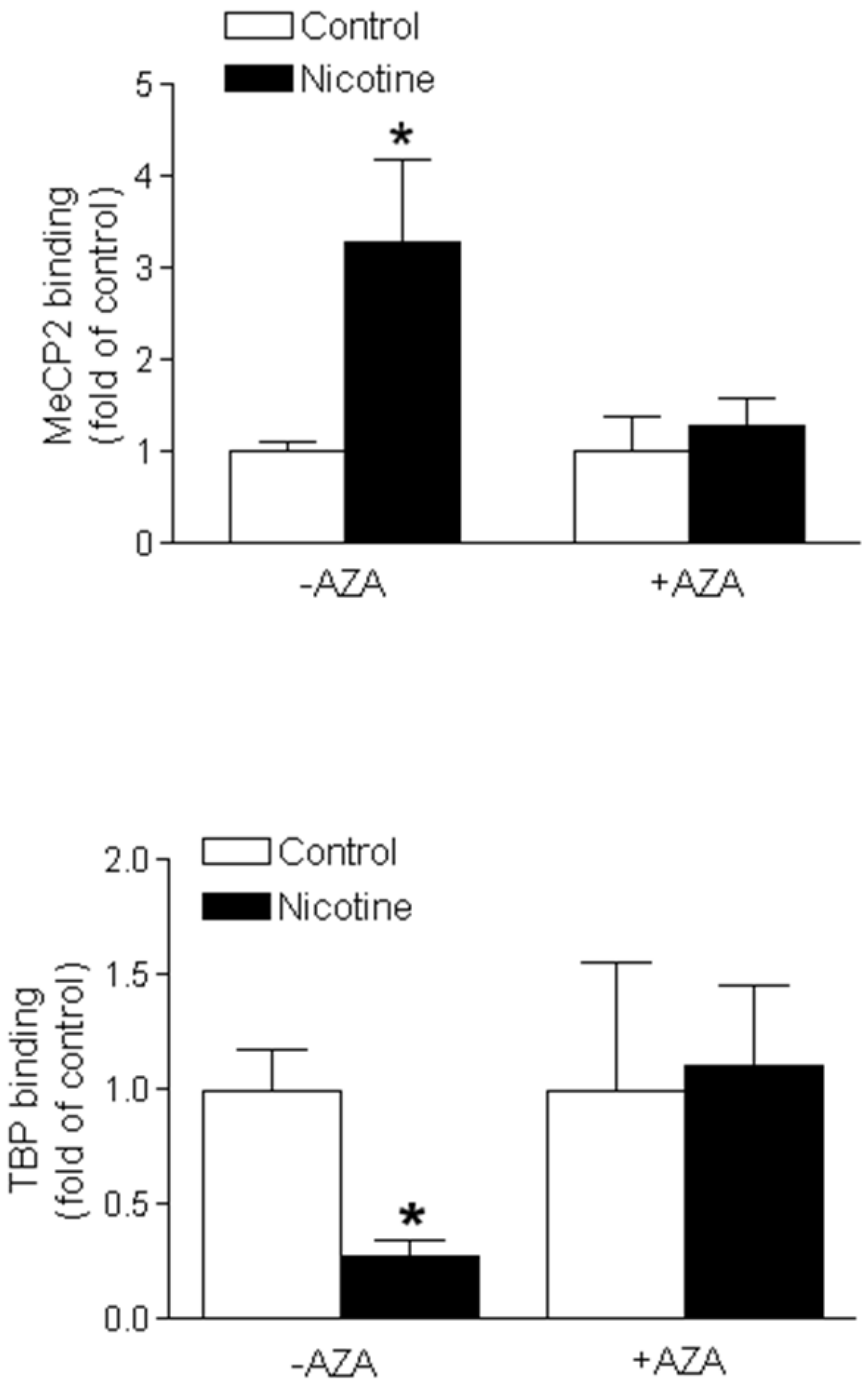

Figure 15. 5-Aza-2'-deoxycytidine reversed nicotine-induced changes in TBP and MeCP2 binding at AT_2R promoter. TBP and MeCP2 binding to the TATA element at AT_2R promoter *in vivo* in the context of intact chromatin was determined with ChIP assays in pup brains from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA). Data are means ± SEM, $n = 5$. *$P < 0.05$ versus control group.

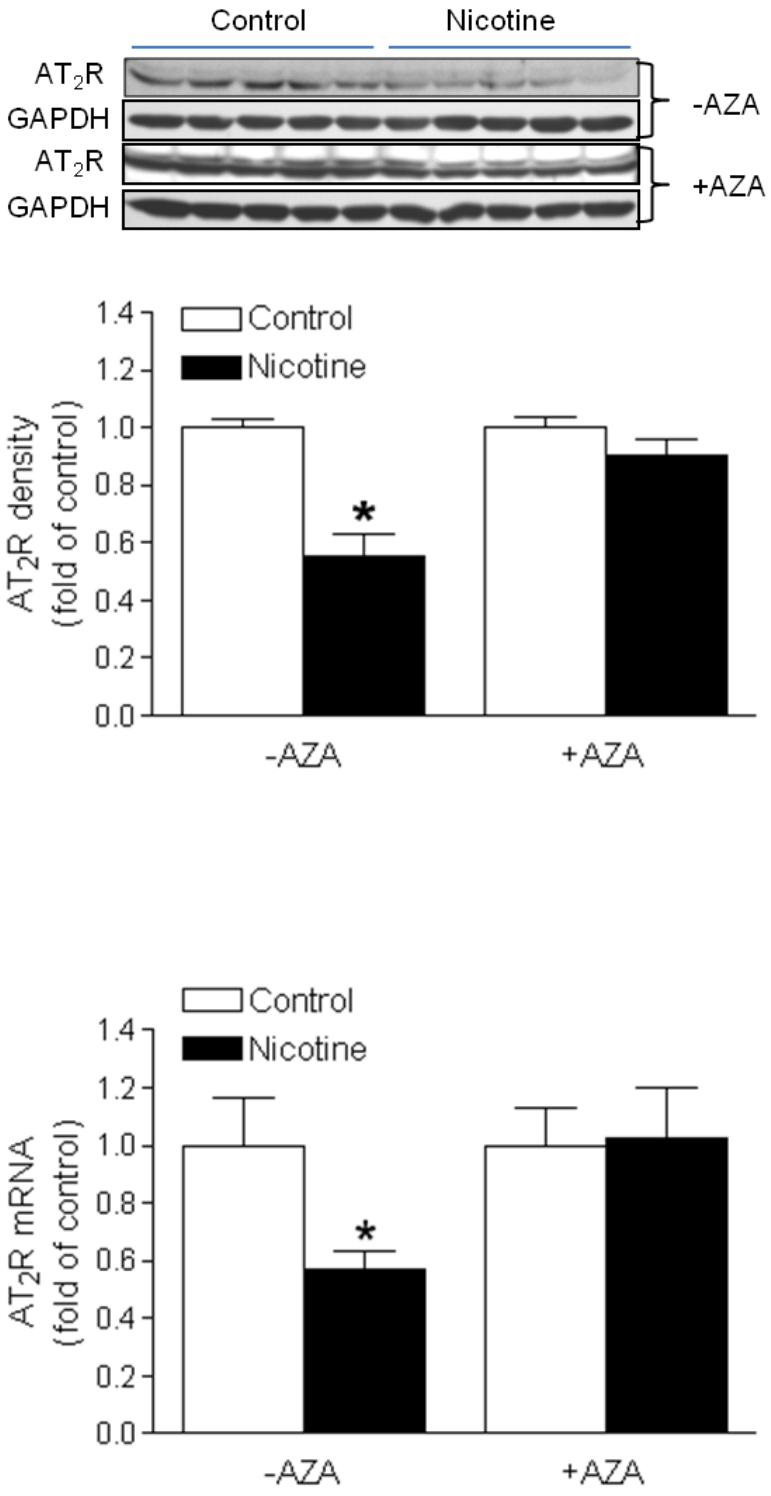

Figure 16. 5-Aza-2'-deoxycytidine restored nicotine-induced down-regulation of AT_2R mRNA and protein expression. AT_2R mRNA and protein abundance was determined in pup brains from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA). Data are means ± SEM, $n = 5$. *$P < 0.05$ versus control group.

5-Aza-2'-deoxycytidine Rescued Nicotine-Induced Vulnerability of HI Injury in Pup Brains

AT_2R 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 AT_2R in the heightened vulnerability of pup brains to HI injury by determining whether 5-aza-2'-deoxycytodine-mediated restoration of AT_2R expression in the developing brain reversed nicotine-induced vulnerability of HI injury in pup brains. As shown in Figure 17, in the absence of 5-aza-2'-deoxycytodine, the nicotine treatment resulted in a significant increase in HI injury in pup brains, which was abolished by 5-aza-2'-deoxycytodine. 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'-deoxycytodine 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'-deoxycytodine were 18.4 ± 0.4 g *vs*. 17.8 ± 0.4 ($P > 0.05$) 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'-deoxycytodine 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.

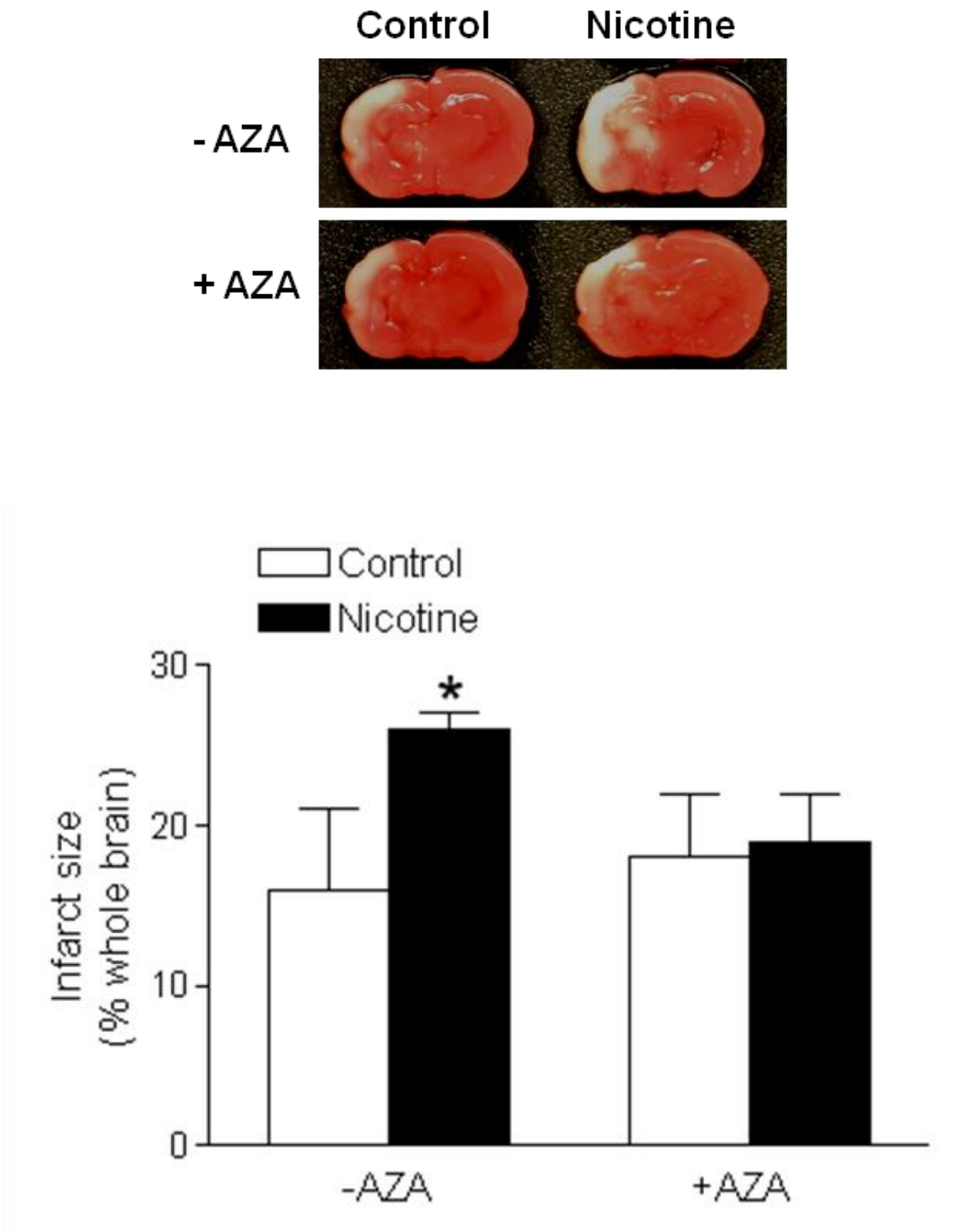

Figure 17. 5-Aza-2'-deoxycytidine rescued nicotine-induced increase in neonatal brain HI injury. Hypoxic-ischemic injury was determined in pup brains from control and nicotine-treated animals in the absence or presence of 5-aza-2'-deoxycytidine (AZA). Data are means ± SEM, $n = 4$ to 7. *$P < 0.05$ versus control group.

Discussion

The present study reveals evidence that heightened methylation of a single CpG_{-52} locus adjacent to the TATA element at AT_2R promoter significantly inhibits the binding activity of TBP and suppresses AT_2R 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'-deoxycytodine blocked nicotine-induced methylation, restored AT_2R 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 AT_2R in a sex-specific manner in the developing brain (Li et al., 2012). Both AT_1R and AT_2R have been implicated in the pathogenesis of neonatal HIE and confer neuroprotective traits. Nicotine treatment significantly repressed expression levels of AT_2R 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 AT_2R agonist CGP42112 reversed the nicotine-induced increase in brain HI injury demonstrated an important role of brain AT_2R repression in programming of enhanced vulnerability of neonatal HIE. However, the underlying molecular mechanisms of perinatal nicotine exposure in repressing AT_2R gene transcription in neonatal brains remained elusive.

Rat AT_2R 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 AT_2R promoter in rat brains. The functional significance of the TATA element in the regulation of rat AT_2R gene activity was demonstrated by the finding that deletion of TATA significantly decreased the AT_2R promoter activity (Xue et al., 2011). The present finding that methylation of a single CpG_{-52} locus 3 bases 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 ε gene (PKCε) promoter and PKCε 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-κB 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 AT_2R promoter in pup brains reveals an important mechanism of site-specific CpG methylation in epigenetic repression of AT_2R gene in the developing brain. 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 AT_2R promoter *in vivo* in pup brains in the context of intact chromatin. 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 at 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 AT_2R 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 AT_2R 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 CpG_{-52} methylation in the nicotine-induced AT_2R 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 CpG_{-52} methylation, rescued TBP binding and restored AT_2R 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 11β-hydroxysteroid dehydrogenase type 2 (11β-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 11β-HSD2 promoter DNA methylation in cell lines, and the decreased level of promoter methylation resulted in a higher expression of the 11β-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 PKCε 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 present investigation provides evidence of a novel mechanism of increased methylation of a single CpG_{-52} 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. 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.

Acknowledgment

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CHAPTOR FOUR

GENERAL DISCUSSION

Perinatal Nicotine Exposure, Oxidative Stress and Aberrant Promoter Methylation

Our present study already demonstrated a close linkage of nicotine induced heightened CpG methylation status at AT_2R promoter, repression of AT_2R gene expression and enhanced vulnerability to neonatal HIE. However, the pathogenic mechanisms underpinning nicotine exposure and hypermethylation status at AT_2R promoter remains poorly understood. Recently, more and more evidence most specifically derived from various carcinogenesis researches revealed the potential association between prolonged oxidative stress exposure and aberrant promoter DNA methylation patterns, which may also shed light on our present study.

In general, oxidative stress indicates a cellular state of which the ROS production outweighs the cell's ability to metabolize or decompose them leading to excessive accumulation of ROS that overwhelms cellular defenses (Ziech et al., 2011). The carcinogenicity of oxidative stress is primarily attributed to the genotoxicity of ROS in diverse cellular processes, such as modification of DNA bases, strand breaks, DNA-protein cross linkages, etc. (Lee and Lee, 2006). Except for causing genetic changes, ROS may also lead to epigenetic alterations which may influence the genome and play a key role in the development of human carcinogenesis (Campos et al, 2007). More specifically, ROS production is correlated to alterations in DNA methylation patterns (Donkena et al., 2010; Ziech et al., 2010). Intriguingly, ROS-induced aberrant DNA

methylation demonstrates bidirectional effects, hypo- or hyper- methylation. Particularly, ROS production induced DNA lesions (such as 8-hydroxyl-2-deoxyguanosine; 8-hydroxyguanine; 8- OHdG [Weitzman et al., 1994; Turk et al., 1995a, 1995b; Kuchino et al., 1987], O^6-methylguanine [Hepburn et al., 1991; Tan and Li,1990] and single stranded DNA [Christman et al., 1995]) have been demonstrated to contribute to decreased DNA methylation by means of interfering with the ability of DNA to function as a substrate for the DNA methyltransferases (DNMTs) and thus resulting in global hypomethylation (Franco et al., 2006), which may induce both genomic instability and activation of protooncogenes, resulting in human carcinogenesis. Alternatively, ROS-induced oxidative stress can also suppress gene expression by mechanisms that involve aberrant hypermethylation of tumor suppressor gene at promoter regions and thus lead to programming of the malignant phenotypes, which is also similar to the observations in our recent studies. For example, one study demonstrated that exposure of hepatocellular carcinoma cells to hydrogen peroxide (H_2O_2) induced hypermethylation of the E-cadherin gene promoter (a tumor suppressor gene) *via* increasing the expression of Snail (a transcription factor that down-regulates the expression of E-cadherin) which then induced methylation at the promoter of E-cadherin gene by recruiting histone deacetylase 1 and DNA methyltransferase 1 (Lim et al., 2008). In fact, many tumor suppressor genes are repressed via ROS-mediated aberrant methylation of CpG island-rich promoter regions (Ushijima and Takada, 2005). Recent genome-wide screening of tumor DNA has also led to the identification of several tumor-suppressor genes displaying oxidative stress-induced alterations in methylation patterns (Ziech et al., 2011), suggesting the critical

roles of oxidative stress exposure induced aberrant promoter methylation patterns of selective genes in human carcinogenesis.

Both human and animal studies have already verified that maternal smoking can induce oxidative damage in both the mother and the fetus (Strakovsky and Pan, 2012). A recent transcriptome analysis of pregnant smokers and non-smokers demonstrated that smoking was associated with altered expression patterns of numerous oxidative stress related genes in peripheral blood, placenta, and cord blood, suggesting that smoking can significantly influence oxidative balance in both maternal and fetal tissues (Votavova et al., 2011). In addition, treatment of fetal explants cultures with cigarette smoke extract resulted in an increase of the markers of oxidative stress and a decrease in antiapoptotic markers (Menon et al., 2011). In rats, maternal nicotine administration enhanced arterial contractions and contributed to hypertension in the offspring, and this was associated with decreased SOD activity, and increased superoxide, MDA, and nitrotyrosine protein levels in the vascular walls of adult offspring (Xiao et al., 2011). Additionally, offspring of rats with prenatal nicotine exposure presented severe pancreatic islet oxidative stress, mitochondrial abnormalities, glucose intolerance, and reduced glucose-stimulated insulin secretion, indicating that prenatal nicotine exposure induced a diabetic phenotype in offspring (Bruin et al., 2008). All of evidence clearly indicates that nicotine exposure may induce extensive oxidative stress in various organs or tissues of both the mother and the fetus.

Thus, it is presumable that, directly or indirectly, perinatal nicotine exposure may also induce sustained oxidative damage in mother and fetus, allowing the fetal brain exposure to prolonged high levels of oxidative stress and contribute to aberrant

promoter DNA methylation patterns of selective genes as observed in carcinogenesis. Study has shown that maternal nicotine exposure treatment can increase norepinephrine levels in fetal rat brains (Onal et al., 2004), which may act as a vital mediator to induce heightened oxidative stress and contribute to hypermethylation status of protective genes at promoter region. In fact, our recent researches have demonstrated maternal nicotine administration induced heightened CpG methylation at Egr-1 and Sp-1 binding sites in PKCε promoter, repressed PKCε gene expression and caused enhanced cardiac vulnerability to ischemia and reperfusion injury in adult rat offspring (Lawrence et al., 2011). Of interest, our subsequent study revealed it is not nicotine itself but increased levels of norepinephrine triggered by nicotine exposure causes CpG methylation of transcription factor binding sites at PKCε promoter in rodent hearts via activating Nox1-dependent reactive oxygen species production (Lawrence et al., 2011; Xiong et al., 2012), suggesting a critical role of prolonged oxidative stress in heightened promoter methylation patterns. Likewise, another research indicates that maternal hypoxia exposure-induced oxidative stress increases CpG methylation of the SP-1 binding sites at PKCε promoter in fetal hearts and H9c2 cells which can be blocked by ROS scavengers (Patterson et al., 2012). Taken together, it is possible that prolonged oxidative stress exposure may be one of the most common pathways to induce aberrant promoter methylation patterns in response to various exogenous and endogenous stressful stimuli including perinatal nicotine exposure and reprogramming of gene expression and vulnerability to postnatal life challenges, which deserves further extensive investigations.

Fetal Stress Reprograms Ischemic-Sensitive Phenotype in the Developing Brain: Other Potential Candidates

In chapter two and three, we have demonstrated that perinatal nicotine exposure epigenetically reprograms expression patterns of AT_1R/AT_2R in the developing brain and causes heightened susceptibility to neonatal hypoxic-ischemic encephalopathy in a sex-specific manner. Given that the potential diverse stressful insults might be experienced by mother and its profound reprogramming effects on the fetal brain structure and function, it is plausible that there are other vital candidates implicated in the complex pathophysiology of ischemic brain injury and contribute to determination of the susceptibility to neonatal HIE in postnatal life, not only limited to AT_1R/AT_2R. Indeed, there is a multitude of promising players may be involved in such pathological processes.

The first promising candidate mediator with potential role in heightened vulnerability of neonatal HIE is glucocorticoid, either cortisol or corticosterone. As described in introduction part, glucocorticoid exerts profound effects on the programming of fetal stress and brain development, particularly their programming effects on the HPA axis activity as well as other important organs and tissues. Sustained overexposure to glucocorticoid down-regulates GR levels in hippocampus, attenuates negative feedback of the HPA axis, permanently resets the activity of HPA axis and enhances basal and stressful glucocorticoid responses in the postnatal life. These will cause the brain to be exposed to chronically high level of glucocorticoid, resulting in aberrant gene regulation and cell behavior and programming of vulnerability of HIE injury. Paradoxically, glucocorticoid shows bidirectional effects on the brain, which may be implicated in both neurodegenerative and neuroprotective processes (Abraham et al., 2001). On the one hand, glucocorticoid may inhibit key nutrients such as glucose uptake, modulate both

excitatory and inhibitory neurotransmission, increase intracellular calcium concentrations, enhance excitotoxicity and induce perturbation of 11β-HSD, which may retard fetal brain growth, delay myelination, promote synapse degeneration and enhance neuronal vulnerability to hypoxic/ischemic insults (Abraham et al., 1996; Doyle et al., 1993; Joels and de Kloet, 1994; Moghaddam et al., 1994; Seckl and Walker, 2001). On the other hand, some studies indicate via modulating calcium currents, increasing synthesis of neurotrophic factors, such as lipocortin-1, basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and decreasing lipid peroxidation, glucocorticoid may be neuroprotective (Flower and Rothwell, 1994; Joels and de Kloet, 1994; Mocchetti et al., 1996; Young and Flamm, 1982). There are experimental findings suggesting that glucocorticoid affects the vulnerability of fetal and neonatal brain to hypoxia-ischemia challenge. However, the results were inconsistent, contradictory, and dependent on experimental protocol, dosage, time, animal age, strains and species (Flavin, 1996; Kauffman et al., 1994; Tombaughet al., 1992; Tuor, 1995, 1997; Whitelaw and Thoresen, 2000). It appears that the concentration and duration of glucocorticoid treatment are the two key factors determining the detrimental or beneficial effects of glucocorticoid in the brain. Exposure to long term and high levels of glucocorticoid enhances neurotoxic effects in brain injury, such as in HIE, whereas physiological or slightly higher (slightly supraphysiological elevated levels in a narrow concentration window) levels of glucocorticoid may confer on the brain protective potential to challenges (Abraham et al., 2001). Although there are some controversial reports in the literature, the notion has been widely accepted that overexposure to glucocorticoid enhances neuronal degeneration (Abraham et al., 2001). Given that most prenatal stress increases both basal and stressful

glucocorticoid levels in offspring mainly via reprogramming of the HPA axis, which may contribute to enhanced vulnerability of neonatal HIE and other challenges, it is plausible that glucocorticoid itself may be a pivotal mediator in such pathophysiological processes. However, such effects may be variable depending on the duration, timing, severity and types of prenatal stresses.

Fetal stress may also reprogram expression patterns of matrix metalloproteinases (MMPs) in the neonatal brain, which contribute to the enhanced vulnerability of HIE. MMPs belong to a family of zinc-dependent proteases that exert pronounced effects in the ECM turnover. These enzymes remodel almost all components of the matrix and play an essential role in cell signaling regulation, cell survival and cell death. MMPs, especially MMP-2, MMP-3 and MMP-9, may target the extracellular matrix of blood vessels, basal lamina, and tight junctions in endothelial cells, increase the permeability of the blood-brain barrier in neuroinflammation due to hypoxia-ischemia, multiple sclerosis and CNS infection, which can result in cytotoxic and vasogenic edema, promote hemorrhagic transformation, induce apoptosis of neurons and oligodendrocytes (Cunningham et al., 2005; Rosenberg, 2009). However, in later stage of such pathology, MMPs play critical roles in tissue repair and remodeling process via inducing angiogenesis and neurogenesis. Growing evidence suggests that overly upregulated activity/expression of MMPs, particularly MMP-2 and MMP-9, are deleterious in the acute phase of stroke. Inhibition of MMPs in the acute phase may reduce the damage to BBB (Gasche et al., 2001). There is a report indicating decreased damage to BBB and reduced infarct size in a focal ischemic MMP-9 knockout model (Asahi et al., 2001). More importantly, a recent study in a neonatal rat HIE model revealed that early

inhibition of MMPs conferred acute and long-term beneficial effects via reducing tight junction proteins degradation, attenuating the permeability of BBB, improving brain edema, and preventing brain atrophy (Chen et al., 2009a). Fetal hypoxia reprograms expression patterns of MMPs in the heart and brain and increases activities/expressions of both MMP-2 and MMP-9 in the neonatal brain (Tong et al., 2010, 2011; Tong and Zhang, 2011). Considering the evident detrimental effects of MMPs in the acute stroke models and other neurological pathophysiologies, it is plausible that altered expression patterns of MMPs by prenatal stress is another important mediator in programming of ischemic-sensitive phenotype and increased susceptibility of HIE in the neonatal brain.

Hypoxia inducible factor-1 (HIF-1), a key regulator in response to cellular hypoxia and oxygen homeostasis (Wang et al., 1995), may be profoundly involved in the programming effects of prenatal stress on the vulnerability to neonatal HIE. Being a heterodimeric transcription factor, HIF-1 consists of an oxygen-sensitive HIF-1α and a constitutively expressed HIF-1β. The normal oxygen level results in a rapid degradation of HIF-1α, but hypoxia can enhance the stability of HIF-1α and promote the transactivation of its target genes. More than 100 HIF-1α targeted genes have been identified up to now, including erythropoiesis, angiogenesis, cell proliferation (IGF-2), glucose metabolism (Glut-1,3), inflammation (COX-2), cell apoptosis (BNIP3, P53), vascular tone and matrix metabolism, etc. (Ke and Costa, 2006). Based on its regulation of a wide spectrum of genes in diverse contexts, the effects of HIF-1α activation may be very complex and variable, to some extent similar to those of glucocorticoids. During brain challenges, such as in hypoxia-ischemia, HIF-1α may be both anti-apoptotic via enhancing the transcription of EPO, VEGF, IGF-2 and GLUT-1, etc., but it also can be

pro-apoptotic by upregulation of factors such as COX-2, BNIP3 and P53 that contribute to cell death (Chen et al., 2009b; Fan et al., 2009). Notably, VEGF promotes the permeability of BBB and enhances brain edema in the acute phase of HI, which is different from its later beneficial effects such as neovascularization (Chen et al., 2009b). The bidirectional effects of HIF-1α in hypoxia may be affected by some factors, such as the duration and severity of hypoxia, and the type of pathological stimuli. Mild hypoxia may predominantly induce anti-apoptotic gene expression, but more sustained and severe hypoxia promotes pro-apoptotic gene expression (Chen et al., 2009b; Fan et al., 2009). In addition, effects of HIF-1α in HI may be cell type specific. In vitro studies suggest that functional loss of HIF-1α may be neuroprotective for astrocyte but enhances neuronal vulnerability to HI injury (Vangeison et al., 2008). Under normal conditions, HIF-1α is essential for normal fetal brain development via the activation of genes such as VEGF because of the relatively lower physiological oxygen level in uterus (Fan et al., 2009; Lee et al., 2001; Trollmann and Gassmann, 2009). In addition to maternal hypoxia, some other prenatal stresses, such as nicotine, cocaine, and ethanol exposure, may also trigger the release of catecholamine, resulting in various degrees of ischemia/hypoxia insult to the fetus and leading to a sustained or episodic upregulation of HIF-1α. Long-term and supraphysiological high levels of HIF-1α in the fetus, combined with its adverse impacts on the developing brain, may persist into the postnatal developmental stage and enhance the vulnerability of neonatal HIE injury.

There are some studies indicating that aberrant development of the monoaminergic system in specific brain regions and/or peripheral organs such as the heart and adrenals also weakens the tolerance to hypoxia/ischemia insults in neonates. It

is well recognized that prenatal nicotine exposure is a major risk factor for SIDS in which defective arousal and cardiorespiratory response adjustment are considered to be the potential mechanisms (Milerad and Sundell, 1993; Slotkin, 1998; Wickstrom, 2007). Prenatal nicotine exposure exerts negative effects on the development of central and peripheral catecholaminergic system by decreasing synthesizing enzymes and reducing synthesis and release of catecholamine in brainstem nucleus, adrenals and heart, which may particularly impact the crucial defensive response to acute stress including hypoxia/ischemia and enhance the vulnerability of neonatal HIE (Slotkin et al., 1987; Wickstrom et al., 2002). These detrimental effects in defensive responses appear to correlate with functional loss of some subtypes of nAChRs via activity-dependent desensitization (Cohen et al., 2002). A recent study in rhesus monkey also reported that prenatal nicotine exposure compromises the brainstem serotonergic pathways, another important neural structure implicated in autonomic function, arousal and cardiorespiratory responses to acute hypoxic/ischemic challenge (Slotkin et al., 2011).

Evidently, the impacts of prenatal stress on fetal and neonatal brain development are very complicated, dynamic, variable and multifaceted, which may also be subtle or drastic, and are profoundly affected by exposure age, duration, protocol, severity and nature of stress stimuli, and genetic traits. The underlying mechanisms of neonatal HIE remain to be further elucidated. In addition to the common potential mediators mentioned above, there are other possible factors that may be involved in programming of the vulnerability of neonatal HIE under different types of prenatal stressor. For example, the decreased expression of some neurotrophic factors, such as BDNF; perturbation of neurotransmitters and their receptors, such as glutamate, GABA and NMDA; enhanced

oxidative stress; dysfunction of mitochondria; and inflammatory factors, may all act as potential mediators to alter the vulnerability of HIE in the neonatal brain (Archer, 2011; Levitt, 1998; Warner and Ozanne, 2010).

Potential Interventional Targets of Neonatal HIE

In our present study, we have demonstrated that both AT_1R and AT_2R are implicated in the pathogenesis of neonatal HIE and confer neuroprotective properties. Besides, administration of 5-Aza, a DNA methylase inhibitor, reverses nicotine exposure induced pathological changes at molecular, cellular and functional levels, suggesting that relatively stable epigenetic modification is still a reversible process and pharmacological epigenetic manipulation may be a feasible approach to treat HIE. However, just as discussed above, diverse potential mediators might be implicated in this complex brain injury process, indicating it is necessary to develop multifaceted, comprehensive and interdisciplinary strategy to overcome this catastrophic brain injury.

Trying to avoid potential stress stimuli during pregnancy is essential for effectively preventing or ameliorating the adverse programming effects on fetal development. Quitting use of ethanol, cocaine and nicotine should be encouraged, which can be further supported by behavioral modifications and counseling strategies. Owing to the lack of solid evidence of its efficacy and safety, NRT should not be readily recommended to pregnant women until carefully weighing its potentially adverse effects on the fetus (Pauly and Slotkin, 2008; Slotkin, 1998). For pregnant women who are strongly indicated for glucocorticoid therapy, the selection of glucocorticoid and administration protocol are vital. Normally, 11β-HSD2 sensitive glucocorticoid should be favored and betamethasone may be preferred to dexamethasone, and low dosage

administration and fewer times of injection may be more beneficial to the fetus based on available clinical studies (Gulino et al., 2009; Heine and Rowitch, 2009; Whitelaw and Thoresen, 2000). It is also important to treat underlying systemic diseases to prevent or attenuate possible placental insufficiency and fetal ischemia/hypoxia and to improve maternal nutrition status with an optimal balanced diet supplying nutrients including various macro and/or micro nutrients when necessary.

A wide variety of emerging evidence has suggested that epigenetic modifications of gene expression patterns exhibit a central role in fetal stress-mediated programming of neurological and cardiometabolic disorders in later life. Predictably, pharmacological manipulations of epigenetic mechanisms present a promising interventional strategy. Indeed, several experimental studies offered exciting results. As mentioned above, programming of the HPA axis provides an important common pathway for the alteration of vulnerability to various pathophysiologies in later life in which epigenetic modification of GR gene expression patterns in hippocampus plays a critical role. Animal studies conducted in high or low maternal LG offspring have revealed that central infusion of a HDAC inhibitor, trichostatin A (TSA) or methyl donor S-adenosyl-methionine (SAM), can reverse the epigenetic modification status in GR promoter region, rescue the binding capacity of NGFI-A to exon 1_7 region, recover GR expression in hippocampus, restore the HPA axis activity, and reverse the increased vulnerability of neurological dysfunction in later life (Weaver et al., 2004, 2005). More importantly, these studies imply that it is still reversible for some gene expression controlled by lasting epigenetic modifications, and enriching postnatal environment, or providing pharmacological interventions may restore long-term aberrant programming effects. In

addition to HDAC inhibitors and DNA methylation inhibitors, other agents, such as plant-derived isoflavone genistein, leptin, folate, fish oil, omega-3 and vitamin D, can alter the corresponding abnormal epigenetic modification status and improve the adverse programming effects caused by prenatal stress (Gregorio et al., 2008; Hypponen et al., 2007; Torrens et al., 2006; Vickers et al., 2008; Wyrwoll et al., 2007). However, up to now, most of epigenetic therapy compounds exert nonspecific modifications on genes and transposable elements, and thus their adverse effects should not be neglected, including inducing or inhibiting other non-responsible genes expression, the potential tumorigenesis and mutagenesis properties, as well as promoting cell-cycle arrest and apoptosis (Karpf et al., 2001; Laird et al., 1995). In general, current epigenetic therapy is still in its infancy.

For the neonates at risk of HIE or already harmed by HIE, prevention and therapy are complex and somewhat frustrating. Timely diagnosis and therapy are crucial but are also very challenging, which greatly affects the final outcomes. If possible, various available clinical techniques, such as advanced neuroimaging, EEG, some reliable biomarkers of brain damage (e.g., S-100, NSE), should be employed to identify and monitor HIE injury in a timely manner (Perlman, 2006; Rees et al., 2011). Despite apparent limitations, moderate hypothermia is the only currently established available therapy for full-term newborns with mild to moderate HIE. A wealth of animal studies have conferred some promising interventional strategies, such as NMDA receptor blockade, NOS inhibition, prevention of apoptosis and free radical formation, administration of neurotrophic factors and growth factors, AT_2 receptor stimulation, as well as early inhibition of MMPs and HIF-1α, all of which should enrich our

understanding of HIE pathophysiology and provide us with more potentially promising therapeutic options (Chen et al., 2009a,b; Perlman, 2006; Rees et al., 2011).

Conclusions

Neonatal HIE is one of the most common causes of brain injury which impacts greatly on human well being but lacks of sufficient effective interventions nowadays; maternal smoking represents the single most widespread perinatal insult in the world associated with adverse pregnancy outcomes for mother, fetus and the newborn; our present study reveals the close linkage between perinatal nicotine exposure and enhanced vulnerability to HIE, which further mirrors the notion of developmental origins of health and disease and also offer a feasible interventional target for prevention or attenuation of HIE. More importantly, our present study reveals its underlying epigenetic mechanism at the molecular level: nicotine exposure causes heightened methylation status of a single CpG_{-52} locus adjacent to TATA element at AT_2R promoter, significantly inhibits binding activity of TBP and recruiting an inhibiting MeCP2 at promoter region, suppresses AT_2R mRNA and protein abundance in male neonatal rat brain and results in enhanced vulnerability to HIE brain injury; inhibiting methylation of CpG_{-52} locus with 5-Aza (ICV) successfully reverses nicotine induced pathological consequences. This not only further enhances our understanding the pathophysiology of HIE but also provides us with another promising interventional targets although epigenetic pharmacological manipulations is just at the beginning and is mainly tested in animal studies. Because of current deficiency in potent and effective therapy, the prognosis and outcome for most neonatal HIE are less than optimal at the best, more exploring and investigations on its epigenetic mechanisms and intervention strategy should be warranted, which may confer

us a further hopeful future in the management of such a catastrophic disease of neonatal HIE.

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