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Fetal stress-mediated hypomethylation increases the brain susceptibility to hypoxic-ischemic injury in neonatal rats

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Abstract

Background and Purpose—Fetal hypoxia increases brain susceptibility to hypoxic-ischemic (HI) injury in neonatal rats. Yet mechanisms remain elusive. The present study tested the hypothesis that DNA hypomethylation plays a role in fetal stress-induced increase in neonatal HI brain injury.

Methods—Pregnant rats were exposed to hypoxia (10.5% O_2) from days 15 to 21 of gestation and DNA methylation was determined in the developing brain. In addition, 5-aza-2'-deoxycytidine (5-Aza) was administered in day 7 pups brains and the HI treatment was conducted in day 10 pups. Brain injury was determined by *in vivo* MRI 48 h after the HI treatment and neurobehavioral function was evaluated 6 weeks after the HI treatment.

Results—Fetal hypoxia resulted in DNA hypomethylation in the developing brain, which persisted into 30-day old animals after birth. The treatment of neonatal brains with 5-Aza induced similar hypomethylation patterns. Of importance, the 5-Aza treatment significantly increased HI-induced brain injury and worsened neurobehavioral function recovery six weeks after the HI-treatment. In addition, 5-Aza significantly increased HIF-1α mRNA and protein abundance as well as matrix metalloproteinase (MMP)-2 and MMP-9, but decreased MMP-13 protein abundance in neonatal brains. Consistent with the 5-Aza treatment, hypoxia resulted in significantly increased expression of HIF-1α in both fetal and neonatal brains. Inhibition of

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Disclosures

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HIF-1α blocked 5-Aza-mediated changes in MMPs and abrogated 5-Aza-induced increase in HI-mediated brain injury.

Conclusion—The results suggest that fetal stress-mediated DNA hypomethylation in the developing brain causes programming of hypoxic-ischemic sensitive phenotype in the brain and increases the susceptibility of neonatal brain to hypoxic-ischemic injury in a HIF-1α-dependent manner.

Keywords

fetal hypoxia; hypoxic-ischemic brain injury; DNA methylation; 5-aza-2′-deoxycytidine; hypoxia-inducible factor 1α

Introduction

Increasing evidence suggests that epigenetic mechanisms are of critical importance in regulating gene expression patterns, profoundly impacting normal brain development and programming of adaptive/maladaptive phenotypes in response to various environmental cues (Choi and Friso, 2010; Dauncey, 2012; Li et al., 2012a; Mehler, 2008). DNA methylation is the most characterized epigenetic mechanism, once considered as an inherently stable mark incapable of rapid change (Godfrey et al., 2007; Moore et al., 2013). Recently, emerging evidence has demonstrated that DNA undergoes rapid methylation and demethylation in the brain by means of distinct mechanisms in an activity-dependent fashion, which is critical for various types of brain function and physiological activity (Borrelli et al., 2008; Guo et al., 2011a, 2011b). However, pathological DNA methylation or demethylation profiles may result in aberrant gene expression and thus contribute to multiple brain pathologies in various neuropsychiatric conditions (Graff et al., 2011; Hwang et al., 2013; Ikegame et al., 2013; Levenson and Sweatt, 2005).

Neonatal hypoxic-ischemic encephalopathy (HIE) is one of major causes of acute brain damage and mortality as well as chronic neurological disability in newborns (Chen et al., 2009b; Vannucci, 2000). Due to the poor understanding of the basic pathogenesis, few universally accepted therapy is available for neonatal HIE except that some studies implied the possible therapeutic effects of moderate hypothermia intervention (Perlman, 2006; Rees et al., 2011). Various candidate mechanisms have been proposed to elucidate the underlying pathogenesis in HIE, of which several molecules were considered as players and promising therapeutic targets, including the glucocorticoid receptor, angiotensin II type 2 receptor, hypoxia-inducible factor 1α (HIF-1α) and matrix metalloproteinase (MMP), *etc*. (Chen et al., 2008, 2009a; Gonzalez-Rodriguez et al., 2014a, 2014b; Li et al., 2012b, 2013). Of great interest, recent studies revealed that adverse intrauterine environment may contribute to aberrant brain development and program a sensitive brain phenotype to neonatal HI insult (Gonzalez-Rodriguez et al., 2014b; Li et al., 2012b; Ma and Zhang, 2015).

Gestational hypoxia is a common stress to the fetal development and increases the risk of neonatal morbidity and mortality (Ma et al., 2014; Ma and Zhang, 2015). Our previous study revealed that chronic fetal hypoxia resulted in increased brain susceptibility to HI injury in neonatal rats (Gonzalez-Rodriguez et al., 2014b), whilst the underlying mechanisms are not

fully elucidated. Herein, we presented a novel finding that gestational hypoxia induced a significant decrease in global DNA methylation and a sustained increase in HIF-1α in the developing brain. Of importance, we demonstrated that DNA hypomethylation in the brain significantly increased HI-induced brain injury in neonatal rats in a HIF-1α-dependent manner and worsened long-term neurobehavioral deficits, which may underlie fetal stress-induced programming of HI sensitive phenotype in the developing brain.

Materials and methods

Experimental animals

Pregnant Sprague Dawley rats were purchased from Charles River Laboratories (Portage, MI). For the hypoxic treatment, pregnant animals were randomly divided into 2 groups: normoxic control and hypoxic treatment (10.5% O_2, days 15 to 21 of gestation), as described previously (Patterson et al., 2010). On day 21 of pregnancy, some animals were killed and brains were isolated from fetuses (E21). Other animals were allowed to give birth, and brains collected from 12-day old (P12) pups and P30 animals. For the 5-aza-2′-deoxycytidine (5-Aza) treatment, P7 pups from normoxic control animals were divided into saline control and 5-Aza-treated groups. Preliminary studies revealed that there were no apparent sex-dependent effects. Thus, pups of mixed males and females were balanced and randomly assigned into the saline control and 5-Aza-treated groups. All animals were kept in a 12-h light/dark cycle, and provided *ad libitium* access to normal rat chow and filtered water. 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.

Hypoxic-ischemic (HI) treatment

P7 pups were administered *via* intracerebroventricular (i.c.v.) injection of 5-Aza (1 mg/kg BW; Sigma) or saline control, as previously reported (Li et al., 2012b, 2013). Briefly, pups were anesthetized with 2% isoflurane and fixed on a stereotaxic apparatus (Stoelting, Wood Dale, IL). An incision was made on the skull surface and bregma was exposed. 5-Aza was injected at a rate of 1 μL/min with a 10 μL syringe (Stoelting) into the right hemisphere following the coordinates relative to bregma: 2.0 mm posterior, 1.5 mm lateral, and 3.0 mm below the skull surface (Han and Holtzman, 2000). The injection lasted 2 min and the needle was kept for additional 5 min before its removal. Brain HI treatment was performed in P10 pups with a modified Rice-Vannucci model, as previously reported (Li et al., 2012b, 2013). In brief, pups were anesthetized with 2% isoflurane, a small incision was made in the right side of neck where the right common carotid artery was exposed and ligated with silk surgical suture. The incision was sutured. After recovery for 1 hour, pups were treated with 8% O_2 for 2 hours. Some pups received a HIF-1α inhibitor, 2-methoxyestradiol (2-ME, i.p., 15 mg/kg) 24 hours before the HI treatment.

MRI scanning *in vivo*

Forty eight hours after the HI treatment, the brain injury was examined by *in vivo* MRI, as previously reported (Ashwal et al., 2014; Fukuda et al., 2013; Ghosh et al., 2011, 2012). Briefly, rat pups were anesthetized with isoflurane (3–4% for induction and 1–2% for

maintaining) and placed in an MRI-compatible stereotactic device to minimize head motion during the imaging. Body temperature was continuously maintained at 36 ± 1°C using a heated water cushion. MRI data of rat pups were collected on a Bruker Avance Biospec 11.7 T (Bruker Biospin, Billerica, MA, USA) using an established protocol. Two imaging data sets were acquired: (1) a T2 weighted imaging (T2WI) (TR 2358 ms, TE 10 ms, FOV 2×2 cm, Matrix 128×128, 20 slices 1 mm thick, 10 echo) and (2) a diffusion weighted imaging sequence (DWI) (TR 2000 ms, TE 50.0 ms, FOV 2×2 cm, Matrix 128×128, 20 slices, b values = 116.96 s/mm^2, 1044.422 s/mm^2). After each imaging session, animals were placed in a heated animal cage and monitored until normal ambulatory responses occurred. MRI data were quantitatively analyzed by Hierarchical Region Splitting (HRS) automated computational software to evaluate volumes for total infarct, necrotic core and penumbra, respectively. As described previously (Ghosh et al., 2011, 2012), lesions were delineated on the T2 maps based on 'a priori' mean threshold MR values (normal tissue< 80 ms, total lesion > 80 ms, penumbra lesion 80 – 170 ms and core lesion > 170 ms, respectively) and the computational results were then overlaid onto correspondent T2 images, where red stands for necrotic core area and blue stands for penumbra area.

Behavioral testing

Neurobehavioral function was determined 6 weeks after the HI treatment by a standard battery of neurobehavioral tests, as described previously (Fukuda et al., 2013; Hartman et al., 2012). This included the tests for motor coordination and balance (rotarod test), spatial learning and memory (water maze test), general activity level (open field test) and anxiety-like behaviors (zero maze test). For the rotarod test, each rat was placed on the rotarod (Rotamex-5, Columbus Instruments, Columbus, OH), which consisted of a rotating spindle 7 cm wide, and had to continuously walk forward to avoid falling. Latency to fall (sec) was recorded as a measure of motor coordination and balance. For the open field test, the open field activity test involved observation for 30 min in opaque open-topped plastic boxes (49 cm long, 35.5 cm wide, 44.5 cm tall). Total distance moved and percent time spent moving were recorded by an overhead camera and analyzed by a computerized tracking system (Noldus Ethovision). For the zero maze test, the elevated zero maze test used an elevated circular track of 10 cm wide with a 100-cm outer diameter and two sets of 30 cm high walls enclosing two opposing quadrants of the track, leaving the other two opposing quadrants open and brightly lit. The animal was placed at the midpoint of one of the open areas of the track and allowed to explore for 5 min. The percentage of time spent in the enclosed (dark) half of the maze was recorded. For the water maze test, the test system consisted of a metal tank 110 cm in diameter, filled with non-toxic opaque water with temperature at 22°C and containing an 11-cm diameter escape platform. Each animal was recorded with an overhead camera, and the Noldus Ethovision computerized tracking system allowed for assessment of variables such as swim distance, latency, proximity to target, swim speed, and relative angular velocity (left/right turn bias).

Brain atrophy evaluation

After the completion of neurobehavioral tests, rats were sacrificed and brains isolated. The weight of both hemispheres was obtained and atrophy of the right hemisphere was determined as right brain weight/left brain weight × 100%.

Western immunoblotting

Proteins were isolated from the right hemisphere of P10 pup brains and Western blots were performed as previously described (Li et al., 2012b). Briefly, 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 HIF-1α (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), MMP-2 (1:1000), MMP-9 (1:1000) and MMP-13(1:500) (Millipore Corporation, Billerica, MA), respectively. After washing, membranes were incubated with secondary horseradish peroxidase conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with Kodak ID image analysis software. Band intensities were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Real-time RT-PCR

RNA was extracted from the right hemisphere of pup brains and HIF-1α mRNA abundance was determined by real-time RT-PCR using an Icycler Thermal cycler (Bio-Rad, Hercules, CA), as described previously (Li et al., 2012b). The HIF-1α primers used were: 5′-tcaagtcagcaacgtggaag-3′ (forward) and 5′-tatcgaggctgtgtcgactg-3′ (reverse). Realtime RT-PCR 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 min, 95°C for 10 min followed by 40 cycles of 95°C for 20 sec, 56°C for 1 minute, and 72°C for 20 sec. Glyceraldehyde-3-phosphate dehydrogenasewas used as an internal reference and serial dilutions of the positive control were performed on each plate to create a standard curve. Polymerase chain reaction was performed in triplicate, and threshold cycle numbers were averaged.

5-^{m}C DNA ELISA

DNA was isolated from brains and global methylation was determined by measuring 5-methylcytosine (5-^{m}C) using a 5-^{m}C DNA ELISA kit (Zymo Research), as described previously (Gay et al., 2015). Briefly, 100 ng of genomic DNA and standard controls provided by the kit were denatured and used to coat the plate wells with 5-^{m}C coating buffer. After incubation at 37°C for 1 h, the wells were washed with 5-^{m}C ELISA buffer and then an antibody mix consisting of anti-5-^{m}C and a secondary antibody was added to each well. The plate was covered with foil and incubated at 37°C for 1 h. After the antibody mix was washed out from the wells with the 5-^{m}C ELISA buffer, a HRP developer was added to each well and incubated at room temperature for 1 h. The absorbance at 405 nm was measured using an ELISA plate reader. The percent 5-^{m}C was calculated using the second-order regression equation of the standard curve that was constructed with negative control and positive controls in the same experiment.

Immunofluorescence staining and con-focal imaging

Under anesthesia, P10 pups were transcardially perfused with ice-cold PBS (pH 7.4) followed by infusion of 4% paraformaldehyde. Brains were then removed and fixed in 4% paraformaldehyde at 4°C for a minimum of 3 days. After dehydrated with 30% sucrose in PBS (pH 7.4), brains were processed to obtain 10-μm tissue slides. As described previously (Li et al., 2012b), immunofluorescence was performed using the following primary antibodies: mouse anti-5mC (1:500; Active Motif, Carlsbad, CA), rabbit anti-HIF-1α (1:50; Santa Cruz) and mouse anti-neuronal nuclei (1:100; Millipore). After blocking with 1% bovine serum albumin in PBS containing 0.5% Triton X-100 for 2 h 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 Alexa Fluor 488 (1:200) and Alexa Fluor 647 (1:200) (Invitrogen, Carlsbad, CA), respectively, for 2 h at room temperature. After 3 washes, sections were stained with Hoechst 33258 (5 μg/mL; Sigma) for 1 min. The sections were then covered with aqueous mounting medium (RD Systems, Minneapolis, MN), visualized and imaged using the Zeiss LSM 710 confocal microscope, as previously described (Li et al., 2012b).

Statistical analysis

Data are expressed as mean ± SEM. Experimental number (n) represents animals from different dams. Statistical significance ($p<0.05$) was determined by one way analysis of variance (ANOVA) followed by Neuman-Keuls post hoc testing or Student *t* test, where appropriate.

Results

Fetal hypoxia and 5-Aza induced global hypomethylation in the developing brain

Figure 1A showed a developmental regulation of DNA methylation levels in the brain. Compared with the fetal brain, there was a significant increase in global methylation in the brain of P30 animals. Of importance, gestational hypoxia resulted in a significant decrease in methylation levels in the fetal brain, which persisted in the postnatal development, inhibiting the increase of methylation during the brain development (Figure 1A). We investigated the role of hypomethylation in fetal stress-induced brain vulnerability to hypoxic-ischemic injury using a broad spectrum DNMT inhibitor, 5-aza-2′-deoxycytidine (5-Aza). As shown in Figure 1B, intracerebroventricular injection of 5-Aza in P7 pups resulted in a comparable decrease in brain DNA methylation as that caused by gestational hypoxia. Con-focal imaging revealed $5^{m}C$ immunoreactivity in both cortex and hippocampus regions with greater immunoreactivity in dentate gyrus compared with CA1 region in the hippocampus in control animals (Figure 1C). In addition, con-focal imaging demonstrated reduced $5^{m}C$ immunoreactivity in both the cortex and hippocampus in 5-Aza-treated animals (Figure 1D).

5-Aza increased HI-induced brain injury and worsened neurobehavioral dysfunction

Pups were treated with 5-Aza (1 mg/kg; i.c.v.) or saline at P7 and the brain HI insult was introduced at P10. The 5-Aza treatment had no significant effects on the body weight (20.46

± 0.33 g *vs*. 19.74 ± 0.40 g; p>0.05), brain weight (1.03 ± 0.02 g *vs*. 1.00 ± 0.02 g; p>0.05) and brain to body weight ratio (0.05 ± 0.00 g/g *vs*. 0.05 ± 0.00 g/g; p>0.05). Compared with the saline group, 5-Aza-treated pups demonstrated a higher mortality during the hypoxic exposure (22% *vs*. 10%). Forty eight hours after the HI insult, *in vivo* MRI and HRS analysis were employed to evaluate HI-induced brain injury. As shown in Figure 2A, HI-induced brain injury was mainly localized to the cortex and hippocampus regions in the ipisilateral hemisphere. Compared to the saline group, the 5-Aza-treated group demonstrated significantly increased brain injury in total infarct size (11 ± 2% *vs*. 4 ± 1%; p<0.05), core necrotic size (4 ± 1% *vs*. 1 ± 0%; p<0.05) and penumbra size (7 ± 1% *vs*. 3 ± 0%; p<0.05) (Figure 2A), consistent with our previous findings in prenatal hypoxia-treated animals (Gonzalez-Rodriguez et al., 2014b). In addition, the 5-Aza treatment resulted in a significant increase in HI-induced brain tissue loss and brain atrophy represented as the decreased right/left brain weight ratio (63 ± 5% *vs*. 89 ± 3%; p<0.05; Figure 2B) six weeks after the HI treatment. Neurobehavioral tests were performed 6 weeks after the HI insult. The 5-Aza treatment significantly increased sensorimotor deficits in the rotarod test (Figure 2C) and decreased activity levels in the open field test (Figure 2D). Animals with 5-Aza treatment also showed diminished time ratio in darkness on the zero maze, suggesting abnormally low anxiety levels or increased risk-taking behaviors (Figure 2E). In contrast, there were no significant differences in the water maze test between the saline control and 5-Aza-treated groups (Figure 2F).

5-Aza altered MMPs expression patterns in neonatal rat brains

MMP-2, −9, −13 play an important role in neuronal plasticity and are implicated in HI-induced neonatal brain injury. We examined whether 5-Aza-induced hypomethylation altered the MMPs expression profiles in the developing brain. As shown in Figure 3, 5-Aza significantly increased both pro- and active-MMP-2 (Figure 3A), and the active form of MMP-9 (Figure 3B) in P10 pup brains. In contrast, 5-Aza caused a significant decrease in both pro- and active-MMP-13 levels in the neonatal brains (Figure 3C).

5-Aza upregulated HIF-1α expression in neonatal rat brains

HIF-1α is a key regulator in oxygen homeostasis and tissue response to hypoxia, which serves as an upstream signal of MMPs. As shown in Figure 3, inhibition of DNA methylation by 5-Aza significantly increased HIF-1α mRNA (Figure 3D) and protein (Figure 3E) abundance in the developing brain, with a much greater effect on HIF-1α mRNA levels. The increased HIF-1α immunoreactivity was observed in both the cortex and hippocampus regions (Figure 3F). Consistent with this finding, our previous study demonstrated that fetal hypoxia resulted in a persistent increase in HIF-1α protein abundance in fetal and P12 pup brains (Gonzalez-Rodriguez et al., 2014b). Figure 4 showed that fetal hypoxia significantly increased HIF-1α mRNA levels in both fetal and neonatal brains, which is closely consistent with the 5-Aza treatment-induced effects in neonatal rat brains.

Inhibition of HIF-1α blocked 5-Aza-induced effects

To determine the role of increased HIF-1α in 5-Aza-induced effects, pups were treated with a HIF-1α inhibitor 2-methoxyestradiol (2-ME, i.p., 15 mg/kg) 24 hours before the HI insult in P10 pups. As shown in Figure 5, 2-ME blocked 5-Aza-induced upregulation of HIF-1α protein (Figure 5A), and reversed the effects of 5-Aza on MMPs expression profiles (Figure 5B, 5C and 5D) in P10 pup brains. Of great importance, the inhibition of HIF-1α abrogated the 5-Aza-mediated increase in HI-induced brain injury in the pups as demonstrated by MRI imaging (Figure 6).

Discussion

The present study confers several novel findings. Firstly, we showed that gestational hypoxia altered normal developmental patterns of DNA methylation, resulting in a persistent global hypomethylation status in the developing brain. We then demonstrated that DNA hypomethylation in the brain resulted in a significant increase in neonatal brain vulnerability to HI-induced injury and worsened subsequent neurobehavioral dysfunction. Furthermore, we revealed that DNA methylation was an important epigenetic mechanism in the regulation of HIF-1α expression and its downstream effectors MMP-2, −9, −13 in the developing brain. Of critical importance, inhibition of HIF-1α blocked hypomethylation-mediated effects in the neonatal brain. Taken together, the present study uncovers a novel mechanism that aberrant global DNA hypomethylation contributes to fetal stress-mediated programming of hypoxic-ischemic sensitive phenotype in the developing brain in a HIF-1α-dependent manner.

Hypoxia is a major stress to the fetal development and may result in irreversible injury in the developing brain and increase risk of central nervous system pathology and long-term neurological complications in offspring (Ma et al., 2014; Ma and Zhang, 2015). Epigenetic mechanisms play a central role in controlling stress-induced enduring effects such as in hypoxia-mediated gene expression (Okano et al., 1999; Yoder et al., 1997). A recent study revealed that short-term, sub-lethal hypoxia exposure caused long-lasting changes to genome wide DNA methylation status in primary hippocampal neuronal cultures, some of which were correlated with expression of neuronal genes involved in networks in regulating growth and development (Hartley et al., 2013). Interestingly, our present study demonstrated a developmental regulation of DNA methylation patterns in the brain and showed a significant increase of global DNA methylation levels in 30-day old pup brains as compared to the fetal brains, which may represent a normally adaptive epigenetic response in postnatal development after birth. Of great interest, gestational hypoxia altered this normal evolving methylation pattern, inducing a significantly decreased global DNA methylation status in fetal and pup brains. For the best of our knowledge, this is the first report about the long-lasting adverse effect of fetal hypoxia on global DNA hypomethylation in the developing brain, which may shed light on further elucidating fetal stress-induced neurodevelopmental pathologies at the epigenetic regulation level.

Properly established and maintained DNA methylation patterns are essential for the normal brain development, cell lineage commitment, and neuronal activities (Guibert et al., 2009; Robertson, 2005; Senner, 2011). Aberrant DNA methylation has been implicated in various

neurodegenerative diseases, as well as in ischemic stroke (Felling and Song, 2014; Hwang et al., 2013; Petrij et al., 1995; Qureshi and Mehler, 2010; Roelfsema et al., 2005; Urdinguio et al., 2009). Consistent with fetal hypoxia, the 5-Aza treatment resulted in a comparable decrease in DNA methylation in the developing brain in the context of normal physiological condition. Of importance, 5-Aza-induced hypomethylation significantly increased HI-induced brain injury in the pups, as well as worsened the adverse outcomes of long-term neurobehavioral function. This is consistent with the previous finding that gestational hypoxia increased the brain susceptibility to HI injury in neonatal rats (Gonzalez-Rodriguez et al., 2014b). These findings suggest that global hypomethylation induced by fetal hypoxia at a critical window of the brain development contributes to programming of an ischemic-sensitive phenotype in the neonatal brain. It has been reported that conditional knockdown of DNMT1 or brain injection of 5-Aza confers neuroprotective effects in adult mice with middle cerebral artery occlusion (Endres et al., 2000, 2001). It is possible that this discrepancy resulted from the differences in stroke models, developmental ages, short *vs*. long-term effects and other experimental procedures, further suggesting the complex diverse effects of DNA methylation on the brain in a highly context-specific manner. The finding that 5-Aza-treated pups demonstrated a higher mortality during the hypoxic exposure is intriguing and suggests a possible involvement of brain hypomethylation in impairing the stress response and increasing mortality of pups in response to acute hypoxic stress.

The present study provides novel evidence that hypomethylation induced a significant up-regulation of both HIF-1α mRNA and protein abundance in the neonatal brains. To our knowledge, this is the first study showing a potential direct effect of DNA methylation in the regulation of HIF-1α mRNA in the developing brain. We demonstrated previously that gestational hypoxia resulted in a persistent increase in brain HIF-1α protein abundance in the fetus as well as P12 pups after birth (Gonzalez-Rodriguez et al., 2014b). We further demonstrated in the present study that fetal hypoxia upregulated HIF-1α mRNA in the fetal and P12 pup brains. These findings are intriguing and suggest that gestational hypoxia-mediated hypomethylation in the developing brain leads to a sustained increase in transcription and expression of HIF-1α in the brain. HIF-1α is mainly regulated by protein degradation *via* either oxygen-dependent or oxygen-independent proteasomal degradation (Brocato et al., 2014; Fan et al., 2009;Koh et al., 2008; Liu et al., 2007; Nguyen et al., 2013). Interestingly, HIF-1α promoter contains high abundance of CpG dinucleotides, which are ready for modification by DNA methylation. Koslowski et al. (2011) reported that inhibition of DNA methylation by 5-Aza enhanced the hypoxia-induced gene expression in tumor cells. In addition, Walczak-Drzewiecka et al. (2010) also observed promoter methylation-dependent suppression of HIF-1α in a hematopoietic cell line, which was de-suppressed by 5-Aza. The present study demonstrated that 5-Aza significantly increased the expression of HIF-1α mRNA in neonatal rat brains, suggesting a novel mechanism of promoter methylation-dependent HIF-1α expression in the developing brain. Alternatively, 5-Aza may also increase a critical component of the SWI/SNF complex, BAF57 that plays a role in the hypoxia-induced expression of HIF-1α (Kenneth et al., 2009). Future studies are warranted to investigate further the direct versus indirect effects of DNA methylation in regulating HIF-1α expression in the developing brain.

Of great interest, the present study revealed that the upregulation of HIF-1α played a critical role in the hypomethylation-induced increase in neonatal brain susceptibility to HI injury. In the brain's response to hypoxia-ischemia, HIF-1α may be either anti-apoptotic or pro-apoptotic (Chen et al., 2009b; Fan et al., 2009). Consistent with the previous studies (Chen et al., 2009b; Helton et al., 2005), 2-ME administration effectively inhibited hypomethylation-mediated upregulation of HIF-1α and more importantly, abrogated the hypomethylation-induced increase in neonatal brain HI injury, further demonstrating the adverse effect of HIF-1α in the pathogenesis of the acute phase of HI injury in the developing brain. Importantly, the finding provides a novel mechanism linking hypomethylation and increased HIF-1α expression in fetal stress-mediated ischemic-sensitive phenotype in the developing brain. In addition, the finding that inhibition of HIF-1α reversed aberrant MMPs expression profiles and abolished the enhanced brain HI injury provides further evidence that MMP-2, −9, −13 may act as downstream effectors of HIF-1α in hypomethylation-induced hypoxic-ischemic-sensitive phenotype in neonatal brains.

Notably, the effects of hypomethylation in the developing brain on long-term neurobehavioral function are complex and highly context-specific. We observed significantly increased deficits in the rotarod test and the open field test in the 5-Aza-treated group but no significant differences in the water maze test between saline control and 5-Aza-treated groups. In addition, 5-Aza-treated animals demonstrated increased abnormality in behaviors in the elevated zero maze test, as indicated by less time spent in the darkness, suggesting decreased anxiety levels or increased abnormal risk-taking behaviors. The underlying mechanisms for the differential effects of hypomethylation on the neurobehavioral function remain to be determined.

In summary, the present study provides novel evidence that abnormal hypomethylation at a critical time window during the brain development enhances the neonatal brain susceptibility to HI injury, demonstrating a key role of epigenetic mechanisms in normal brain development and programming of hypoxic-ischemic-sensitive phenotype in response to fetal stress. Of critical importance, the present finding demonstrates a novel mechanism of increased HIF-1α expression linking hypomethylation and pathophysiological consequences in the developing brain. The finding of potential epigenetic regulation of HIF-1α expression in the developing brain at the transcription level provides new insights into our understanding of hypoxia-independent regulation of HIF-1α in various physiological and pathological conditions. Although it may be difficult to translate the present findings directly into humans, the possibility that fetal stress-mediated DNA hypomethylation in the developing brain results in programming of an ischemic-sensitive phenotype with a consequence of increased brain vulnerability to hypoxic-ischemic injury provides a mechanistic understanding worthy of investigation in humans.

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Highlights

- Fetal hypoxia induces global DNA hypomethylation in offspring brain.
- DNA hypomethylation increases HIF-1α expression in the developing brain.
- DNA hypomethylation sensitizes brain to HI injury in a HIF-1α-dependent manner.
- Hypomethylation contributes to fetal hypoxia enhanced susceptibility to HIE.

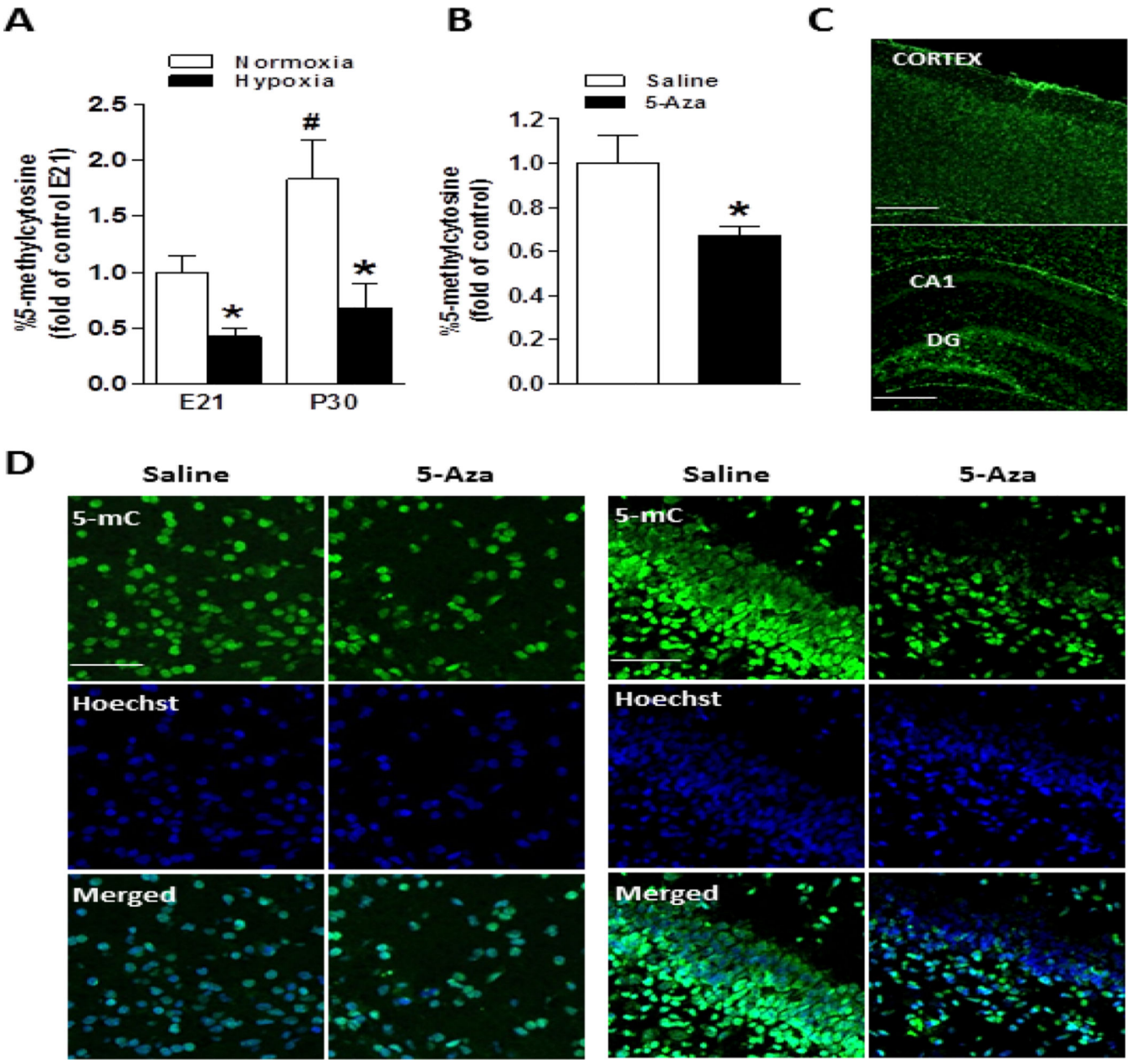
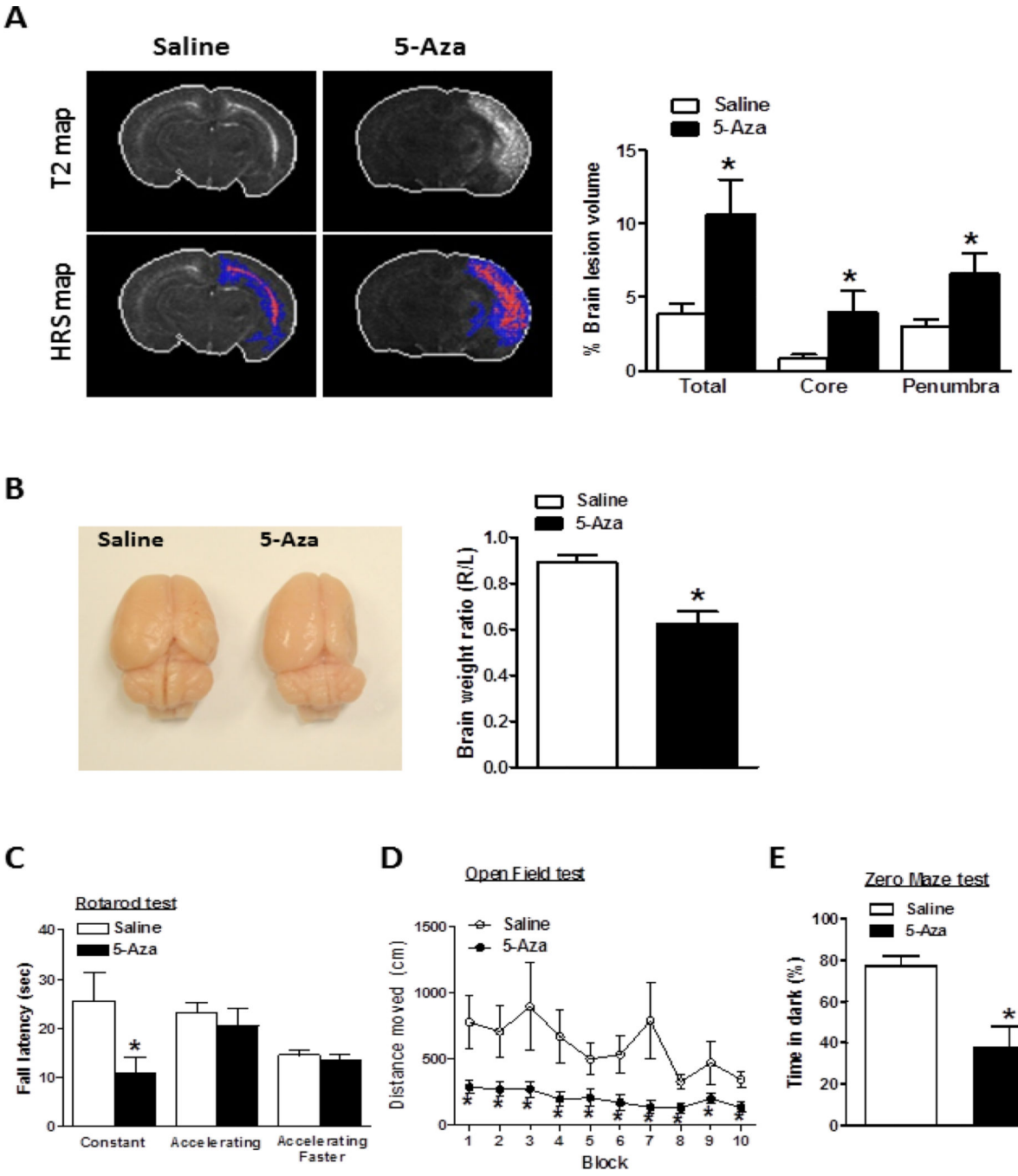


Figure 1. Effects of fetal hypoxia and 5-Aza on DNA methylation in developing rat brains

A. Fetal hypoxia was conducted from day 15 to 21 of gestation and brains were collected from fetus (E21) and 30-day old (P30) pups. DNA methylation levels were determined by 5-^{m}C ELISA. Data are means ± SEM. n = 4 to 6. *p<0.05, hypoxia versus normoxia control; #p<0.05, P30 versus E21 in normoxia control. **B**. 5-Aza (1 mg/kg BW; i.c.v.) or saline was administered in P7 pups and brains were collected from P10 pups. DNA methylation levels were determined by 5-^{m}C ELISA. Data are means ± SEM. n = 4 to 6. *p<0.05, 5-Aza versus saline control. **C**. 5^{m}C immunoreactivity in P10 pup brains. Scale bars: 200 μm. **D**. 5^{m}C immunoreactivity in the cortex and hippocampus in P10 pups with 5-Aza or saline treatment. Scale bars: 50 μm.



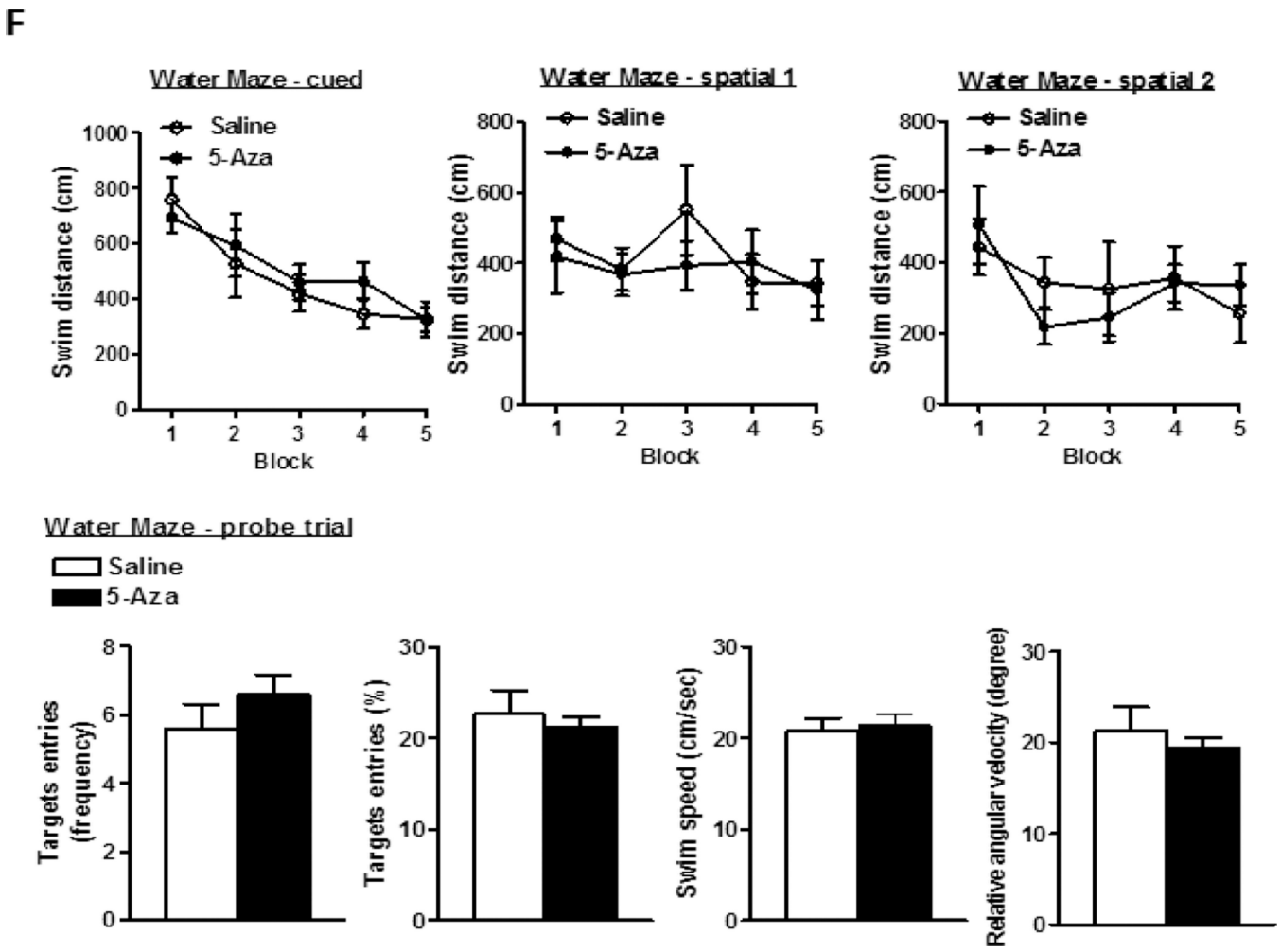


Figure 2. Effects of 5-Aza on neonatal HI brain injury and long-term neurobehavioral deficits

5-Aza (1mg/kg BW; i.c.v.) or saline was administered in P7 pups and the HI treatment was conducted in P10 pups. **A**. Brain injury was determined by MRI *in vivo* imaging 48 h after the HI treatment. Upper lane shows the representative T2 maps and lower lane shows the representative images with HRS analysis overlaid on correspondent T2 images. Red area represents core necrotic lesion and blue area represents penumbra lesion. Lower bar graph shows the brain lesion volume. **B**. Brain atrophy was determined six weeks after the HI insult. **C - F**. Neurobehavioral tests were conducted 6 weeks after the HI injury. Data are means ± SEM. n = 9 to 13.*$p<0.05$, 5-Aza versus saline control.

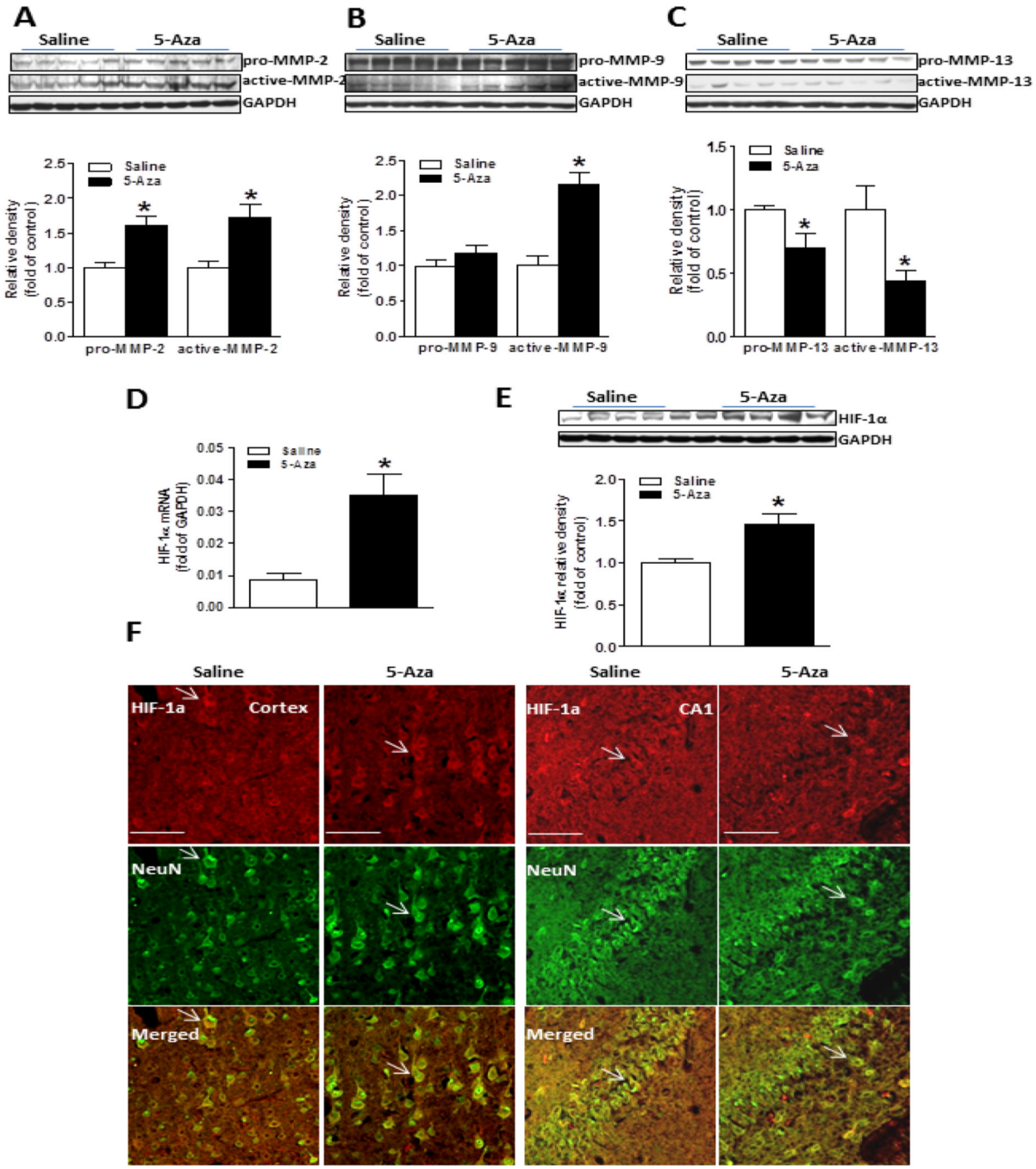


Figure 3. Effects of 5-Aza on expression profiles of MMPs and HIF-1α in neonatal rat brains

5-Aza (1 mg/kg BW; i.c.v.) or saline was administered in P7 pups and brains were collected from P10 pups. Pro- and active-MMP proteins and HIF-1α protein abundance were determined by Western immunoblotting. HIF-1α mRNA was determined by real-time qPCR. HIF-1α immunoreactivity was examined by con-focal imaging. **A**. MMP-2. **B**. MMP-9. **C**. MMP-13. **D**. HIF-1α mRNA. **E**. HIF-1α protein. **F**. HIF-1α immunoreactivity. Data are means ± SEM. n = 5.*p<0.05, 5-Aza versus saline control.

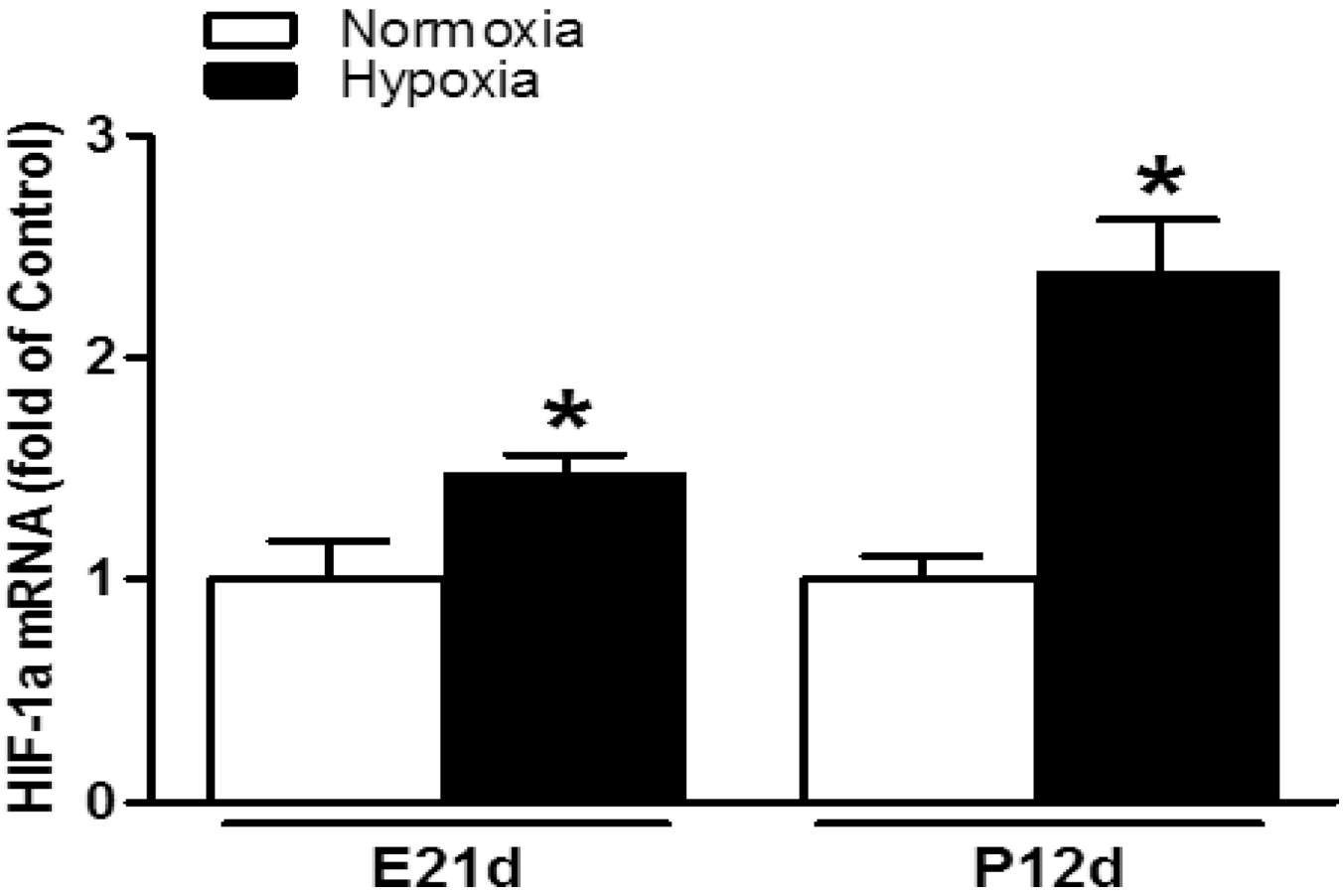


Figure 4. Effects of fetal hypoxia on HIF-1α mRNA in fetal and neonatal rat brains

Fetal hypoxia was conducted from day 15 to 21 of gestation and brains were collected from fetus (E21) and 12-day old (P12) pups. HIF-1α mRNA was determined by real-time qPCR. Data are means ± SEM. n = 6. *p<0.05, hypoxia versus normoxia control.

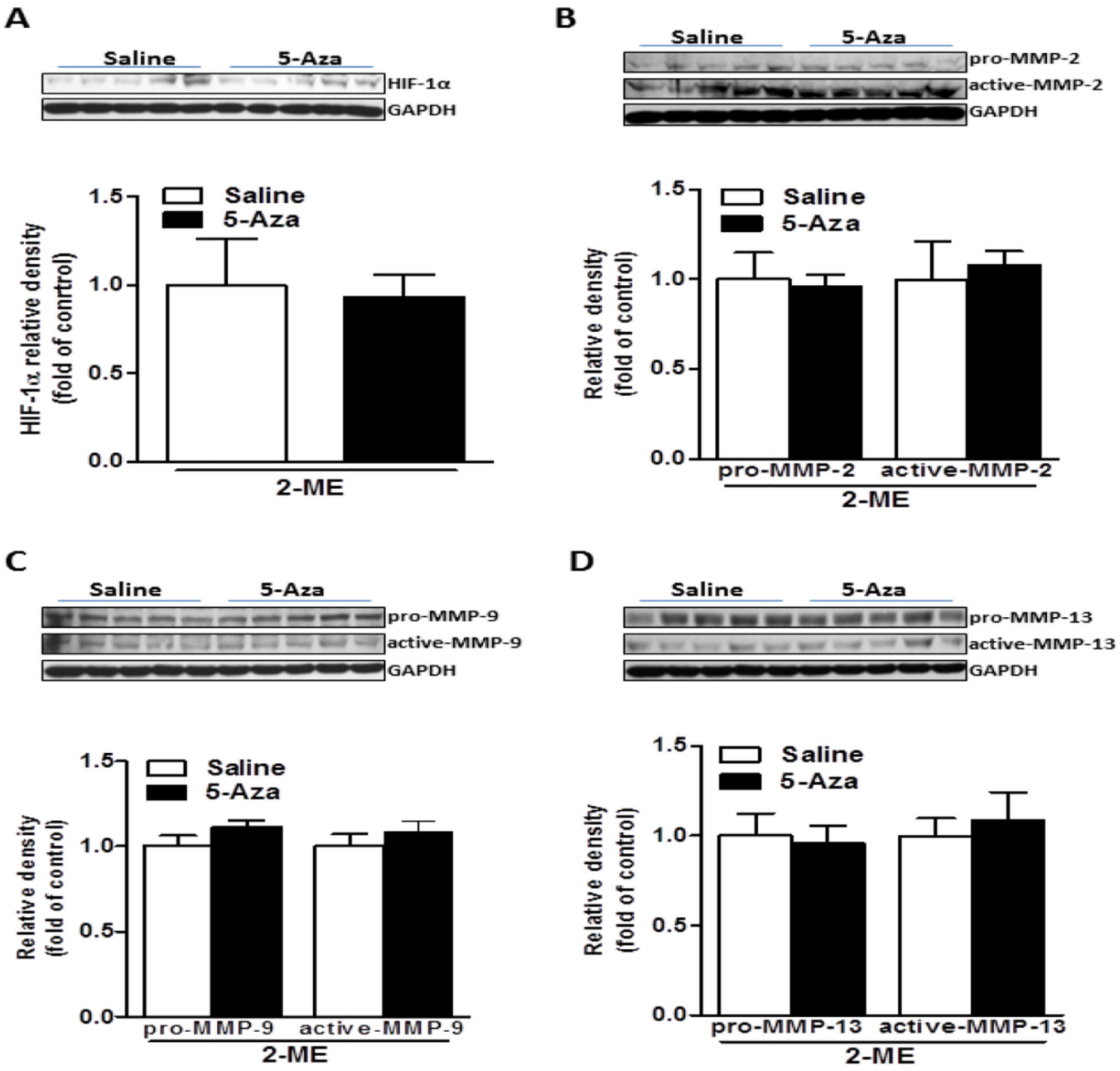


Figure 5. Effects of 2-methoxyestradiol (2-ME) on 5-Aza-induced expression profiles of HIF-1α and MMPs in neonatal rat brains

5-Aza (1 mg/kg BW; i.c.v.) or saline was administered in P7 pups. 2-ME (15 mg/kg BW; i.p.) was administered in P9 pups. Brains were collected from P10 pups. Protein abundance was determined by Western immunoblotting. **A**. HIF-1α. **B**. MMP-2. **C**. MMP-9. **D**. MMP-13. Data are means ± SEM. n = 5.

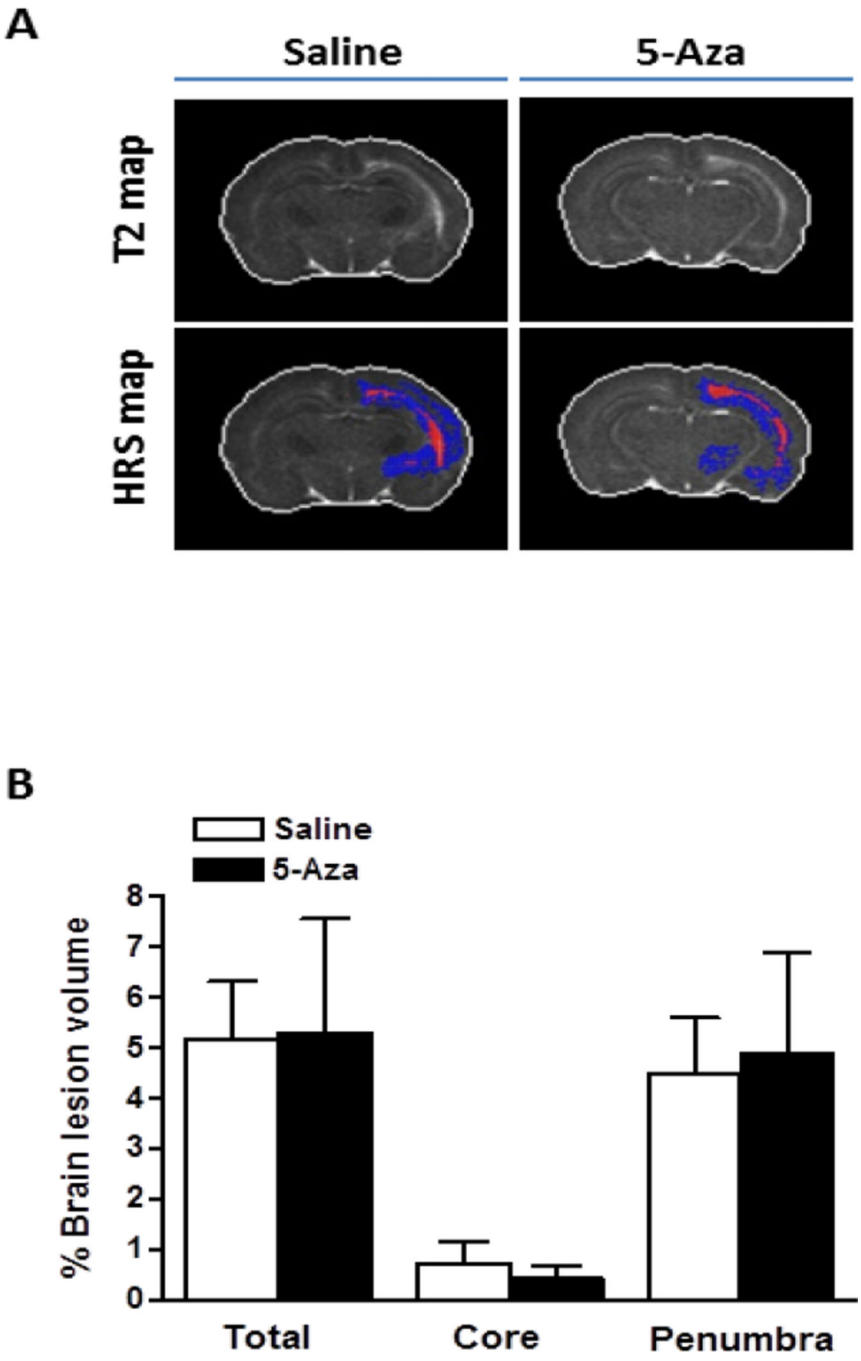


Figure 6. Effects of 2-methoxyestradiol (2-ME) on 5-Aza-enhanced neonatal HI brain injury

5-Aza (1 mg/kg BW; i.c.v.) or saline was administered in P7 pups. 2-ME (15 mg/kg BW; i.p.) was administered in P9 pups. The HI treatment was conducted in P10 pups, and brain injury was determined by MRI *in vivo* 48 h after the HI treatment. **A**. Upper lane shows the representative T2 maps and lower lane shows the representative images with HRS analysis overlaid on correspondent T2 images. Red area represents core necrotic lesion and blue area represents penumbra lesion. **B**. Brain lesion volume. Data are means ± SEM. n = 6.