Uteroplacental insufficiency affects epigenetic determinants of chromatin structure in brains of neonatal and juvenile IUGR rats

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Uteroplacental insufficiency affects epigenetic determinants of chromatin structure in brains of neonatal and juvenile IUGR rats. Physiol Genomics 25: 16–28, 2006. First published December 27, 2005; doi:10.1152/physiolgenomics.00093.2005.—Intrauterine growth retardation (IUGR) increases the risk of neuroendocrine reprogramming. In the rat, IUGR leads to persistent changes in cerebral mRNA levels. This suggests lasting alterations in IUGR cerebral transcriptional regulation, which may result from changes in chromatin structure. Candidate nutritional triggers for these changes include altered cerebral zinc and one-carbon metabolite levels. We hypothesized that IUGR affects cerebral chromatin structure in neonatal and postnatal rat brains. Rats were rendered IUGR by bilateral uterine artery ligation; controls (Con) underwent sham surgery. At day of life 0 (d0), we measured cerebral DNA methylation, histone acetylation, expression of chromatin-affecting enzymes, and cerebral levels of one-carbon metabolites and zinc. At day of life 21 (d21), we measured cerebral DNA methylation and histone acetylation, as well as the caloric content of Con and IUGR rat breast milk. At d0, IUGR significantly decreased genome-wide and CpG island methylation, as well as increased histone 3 lysine 9 (H3/K9) and histone 3 lysine 14 (H3/K14) acetylation in the hippocampus and periventricular white matter, respectively. IUGR also decreased expression of the chromatin-affecting enzymes DNA methyltransferase 1 (DNMT1), methyl-CpG binding protein 2 (MeCP2), and histone deacetylase (HDAC1) in association with increased cerebral levels of zinc. In d21 female IUGR rats, cerebral CpG DNA methylation remained lower, whereas H3/K9 and H3/K14 hyperacetylation persisted in hippocampus and white matter, respectively. In d21 male rats, IUGR decreased acetylation of H3/K9 and H3/K14 in these respective regions compared with controls. Despite these differences, caloric, fat, and protein content were similar in breast milk from Con and IUGR dams. We conclude that IUGR results in postnatal changes in cerebral chromatin structure and that these changes are sex specific.

Barker’s fetal origins of adult disease hypothesis; zinc; DNA methylation; histone deacetylase; histone acetylation

INFANTS IN BOTH DEVELOPED and undeveloped countries suffer from intrauterine growth retardation (IUGR). Epidemiologic issues for IUGR infants include poor neurodevelopmental outcome and problems associated with neuroendocrine reprogramming (35, 36, 65, 89, 104). These issues are examples of Barker’s “fetal origins of adult disease hypothesis,” which proposes that fetal adaptation to a deprived intrauterine milieu leads to persistent changes in cellular biology and systemic physiology (3). Causes of IUGR, such as pregnancy-induced hypertension, transiently deprive the fetus by reducing the delivery of substrate to the fetus. As a result, the fetus suffers moderate fetal hypoxia, acidosis, hypoglycemia, hypoinsulinemia, and decreased levels of growth factors and amino acids, which initiates a series of adaptations that ensure immediate survival (18–21).

Uteroplacental insufficiency caused by bilateral uterine artery ligation in the pregnant rat subjects the rat fetus to a intrauterine environment that is similar to the human condition (77–79, 96, 97). The IUGR rat recovers quickly from the initial insult during the perinatal period and appears to be metabolically normal until it develops insulin resistance in young adulthood (88, 94). Because of presumed protective mechanisms such as the “diving reflex,” the brain of the IUGR rat does not experience the same magnitude of insult as other tissues; however, the IUGR brain is affected by the altered intrauterine environment, as evidenced by altered perinatal mRNA levels of key apoptotic proteins as well as by persistent changes in mRNA levels of genes related to energy metabolism. These changes last beyond the period of recovery and occur before the onset of insulin resistance (57, 58).

The molecular mechanisms underlying these changes in mRNA levels are unknown, but the aforementioned findings suggest an alteration in transcriptional regulation that is relatively persistent. Epigenetic modifications of chromatin structure cause persistent alterations in transcriptional regulation and involve processes such as DNA methylation and histone acetylation.

Methylation of CpG dinucleotides is an important epigenetic mechanism that alters chromatin structure and thereby influences processes such as DNA replication and DNA transcription (12, 71, 76). DNA methylation inversely correlates with histone acetylation, which alters histone-DNA contact and affinity (40, 63, 76). Considering the ubiquitous nature of DNA methylation and histone acetylation in genome control pathways, these phenomena are possible mechanisms through which uteroplacental insufficiency could initiate a “metabolic imprint.” By permanently altering cerebral chromatin structure and subsequently affecting patterns of gene expression, IUGR could contribute to the pathogenesis of postnatal complications.
such as neurodevelopmental delay and neuroendocrine reprogramming (98).

We therefore hypothesized that uteroplacental insufficiency in the rat would alter cerebral DNA methylation and histone acetylation. To prove this hypothesis, we measured DNA methylation, as well as histone acetylation, in both control and IUGR rat brains at day of life 0 (d0) and day of life 21 (d21). Because of the differences we found at d0, we used two approaches to identify cellular mechanisms through which these differences occur. First, we measured and localized expression of proteins involved in DNA methylation [DNA methyltransferase 1 (DNMT1); methyl-CpG binding protein 2 (MeCP2)] and histone acetylation [histone deacetylase (HDAC)] in control and IUGR d0 rat brains; d0 cerebral HDAC activity was also measured.

Second, we measured cerebral levels of trace minerals, with a particular interest in zinc, and one-carbon metabolites in control and IUGR d0 rat brains. Cerebral zinc levels are relevant because zinc deficiency leads to IUGR, and zinc is a cofactor for many of the enzymes involved in determining chromatin infrastructure, including DNMT1 and HDAC1 (26, 69, 74, 86). We investigated the effect of uteroplacental insufficiency on whole cerebral one-carbon metabolism because it potentially contributes to the regulation of genome-wide DNA methylation via S-adenosylmethionine (SAM) and S-adenosyl-homocysteine (SAH) levels.

METHODS

Animals. All procedures were approved by the University of Utah Chancellor’s Animal Research Committee and are in accordance with the American Physiological Society’s guiding principles (2). All surgical methods have been described previously (47–58, 66, 88, 94).

Briefly, pregnant rats on day 19 of gestation were anesthetized with surgical methods have been described previously (47–58, 66, 88, 94). Animals were delivered by cesarean section (dH2O) with a final wash in PBS. Sections were then incubated in a 2-mercaptoethanol) at 55°C for 30 min and then was reprobed with stripping buffer (62.5 mM Tris 1:400 (Cell Signaling, Beverly MA), anti-acetyl-H3/K14 at 1:5,000, anti-acetyl-H3/K9 at 1:1,000 (Upstate Cell Signaling), anti-acetyl-H4 at 1:1,000 (Upstate Cell Signaling), anti-acetyl-H3 lysine 18 at 1:500, and anti-histone H3 at 1:2,000 (Cell Signaling, Beverly MA). Blocking was carried out with freshly prepared PBS plus 3% nonfat milk. After washing, the membrane was incubated overnight with primary antibodies diluted in PBS-milk. Primary antibodies included anti-acetyl-H3 at 1:1,000 (Upstate Cell Signaling, Lake Placid, NY), anti-acetyl-H4 at 1:1,000 (Upstate Cell Signaling), anti-acetyl-H3/K9 at 1:400 (Cell Signaling, Beverly MA), anti-acetyl-H3/K14 at 1:5,000, anti-acetyl histone 3 lysine 18 at 1:500, and anti-histone H3 at 1:2,000 (Upstate Cell Signaling). Separate membranes were used for each primary antibody (histone and nonhistone). After a result was obtained from acetylated H3, the same membrane was stripped with stripping buffer (62.5 mM Tris-HCl pH 6.7, 2% SDS, 100 mM 2-mercaptoethanol) at 55°C for 30 min and then was reprobed with total H3. Secondary antibodies conjugated with horseradish peroxidase (HRP) were incubated for 1 h at room temperature. Signal was detected with enhanced chemiluminescence (ECL) according to the manufacturer’s instructions (Amersham, Little Chalfont, UK). The amount of site-specific acetylated H3 was quantified relative to the amount of total H3 in the sample.

Immunohistochemistry analysis. Immunohistochemistry was used to localize the differences in H3 acetylation occurring between IUGR and Con groups, as well as DNMT1, MeCP2, and HDAC1 expression. In brief, coronal sections of embedded brain tissue were deparaffinized and rehydrated in a graded series of ethanol and distilled H2O (dH2O) with a final wash in PBS. Sections were then incubated in a 3% H2O2 solution for 30 min at room temperature to quench endogenous peroxidase activity. After a brief PBS wash, slides were subjected to an antigen retrieval procedure (Biogenex Laboratories, San Ramon, CA), in which the slides were put in a slide tray with 10 mM citrate solution, pH 6.0, placed in a microwave oven, and heated on high power for 165 s and on low power for 8 min, after which they were cooled to room temperature. Slides were then rinsed with tap water, washed in PBS for 10 min, and incubated in a blocking buffer (2% normal goat serum, 2% bovine serum albumin, 0.8% Triton X-100, 0.2% nonfat dry milk in PBS) at room temperature for 1 h. They were then probed overnight at 4°C in a humidified
chamber with DNMT1, MeCP2, or HDAC1, acetyl-H3/K14, or acetyl-H3/K9 antibodies at 1:100 to 1:1,000 dilution in blocking buffer. The next day, sections were washed in PBS containing 0.2% Tween 20 three times and exposed to biotinylated goat anti-rabbit antibody for 1 h. After exposure to a Vectastain avidin-biotin complex mixture for 1 h, slides were washed in PBS for 15 min, stained with diaminobenzidine (Sigma), counterstained with hematoxylin, dehydrated, and coverslipped with Cytoseal 60 ( Stephens Scientific, Kalamazoo, MI).

**Transmission electron microscopy.** Transmission electron microscopy was performed to visualize chromatin ultrastructure. The analysis focused on the hippocampus because this region is vulnerable to IUGR insult and contains a high density of hormone receptors (42, 59). Brains of d0 rat pups were fixed for transmission electron microscopy. Briefly, the brains were placed in 2.5% glutaraldehyde-1% paraformaldehyde in buffer (pH 7.4, 310 mosmol/kgH2O, 4°C for 24 h), after which the hippocampus was isolated. The principles of systematic, uniform, and random sampling were used to collect tissue blocks of hippocampus (1 mm³ per hippocampal region per brain) (7). The tissue blocks were postfixed in 1% osmium tetroxide, dehydrated in a graded acetone series, and infiltrated and embedded in epoxy resin. Thin sections (80-nm thickness) were cut with the aid of a diamond knife, counterstained with uranyl acetate and lead citrate, and analyzed with a Hitachi H-7100 transmission electron microscope. Neurons in the hippocampal region were photographed at the same magnification in the upper left corner of each grid square for an entire thin section per tissue block. Thin sections from four tissue blocks were photographed per brain. Image analysis for nuclear dimensions was performed on digitized images. Nuclear average, perimeter, and maximum and minimum diameters (measured perpendicularly) were determined with Bioquant Image Analysis software (Bioxquant, Nashville, TN). Ten nuclei per thin section were measured.

**RNA isolation and real-time RT-PCR.** Brain mRNA levels of DNMT1, HDAC1, and McP2 were measured by real-time RT-PCR, as previously described (54, 81). In brief, total RNA was extracted from d0 brains with an RNeasy Minute Kit (Qiagen, Valencia, CA), treated with DNase I (Ambion, Austin, TX), and quantified by ultraviolet absorbance (16). Sample integrity was confirmed by gel electrophoresis. The probe and primers were designed with Primer Express (PE Applied Biosystems, Foster City, CA) with a reporter dye FAM and a TAMRA quencher dye (Table 1). cDNA was synthesized from 2 μg of DNase-treated total RNA. cDNA- and gene-specific probe and primers were added to Taqman universal PCR master mix (PE Applied Biosystems), and samples were run on an ABI Prism 7900. Real-time RT-PCR quantification was then performed with the Taqman GAPDH as an internal control. Before the use of GAPDH as an internal control, the validity of using GAPDH as an internal control. Relative quantification of PCR products was based on value differences between the target and GAPDH control by the comparative threshold cycle method (Taqman Gold RT-PCR master mix, PE Applied Biosystems). Cycle parameters were 50°C for 2 min, 95°C for 10 min, and then 40 cycles at 95°C for 15 s and 60°C for 60 s. For each set of reactions, samples were run in triplicate.

**Protein isolation and Western blotting.** Protein levels of DNMT1, McP2, and HDAC1 were measured in d0 Con and IUGR brains. In brief, whole brains were homogenized in ice-cold lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 0.25% Na-deoxycholate, 1% Igepal CA-630) with protease inhibitors and 0.1 M PMSF. After centrifugation (10,000 g) at 4°C for 15 min, supernatants were stored at −80°C until use. Protein concentrations were determined by the BCA method (Pierce, Rockford, IL). Proteins were separated by 10% SDS-PAGE ready gels (Bio-Rad, Hercules, CA) and transferred to nitrocellulose membranes in standard transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). After the membranes were blocked with 5% milk in Tris-buffered saline (TBS) for 1 h, bound proteins were exposed to specific antibodies against DNMT1, HDAC1, or McP2 (rabbit polyclonal, Santa Cruz Biotechnology) overnight at 4°C. After extensive washing in TBS with 0.1% Tween 20, a 1:2,000 dilution of goat anti-rabbit HRP secondary antibody (Cell Signaling Technology) was applied, and incubated for 1 h at room temperature. After extensive washing, signals were detected with Western Lightning ECL (PerkinElmer Life Sciences) and Biomax film (Amersham) and quantified by densitometry or by quantification on a Kodak Image Station 2000R (Eastman Kodak/SIS, Rochester, NY).

**Nuclear protein extracts preparation.** Nuclear protein was isolated to provide substrate for HDAC nuclear activity assays. Whole brains were ground under liquid nitrogen. Tissue that was used to prepare nuclear extracts for the HDAC activity assay was first washed with ice-cold PBS to lyse red blood cells. The ground tissue was resuspended in 5 ml of ice-cold PBS and then centrifuged (2,000 g) for 5 min. The washing step was repeated until all color was removed. The ground tissue was resuspended in 5 volumes of buffer A (in mM: 1 DTT, 0.5 PMSF, 10 KCl, 10 HEPES, pH 7.9, 1.5 MgCl2) plus protease inhibitors, incubated for 15 min on ice, and then centrifuged (2,000 g) for 5 min. The supernatant was removed by aspiration, and the tissue pellet was resuspended in 2 volumes of buffer A. The tissue was disrupted with a dounce homogenizer, using the tight pestle. Lysis was checked for every 10 strokes with Trypan blue (39). The lysate was centrifuged (1,000 g) for 10 min. The nuclear pellet was resuspended in 0.5 ml of buffer C [in mM: 1.5 MgCl2, 1 DTT, 420 NaCl, 0.5 PMSF, 0.2 EDTA, 20 HEPES, pH 7.9, with glyceral 25% (vol/vol)] plus protease inhibitors, incubated at 4°C for 30 min, and then centrifuged at 17,000 g for 15 min. The supernatant was collected and stored at −80°C.

**HDAC enzyme activity assay.** An HDAC activity assay kit (Abcam, Cambridge, MA) was used to measure HDAC activity in the d0 brain nuclear extracts as described by the manufacturer. Fifty micrograms of nuclear protein was added to each well of a 96-well plate. Two microliters of the HDAC inhibitor Trichostatin A at a final concentration of 1 mg/ml was added to the negative control wells.

**Mineral analysis.** Whole brain zinc, iron, and copper levels were measured as previously described (43). Briefly, brain tissue was minced and digested with concentrated nitric acid and wet-ashed with

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**Table 1. PCR primers for real-time PCR**

<table>
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<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<td>CATGTTGCACATGACACT</td>
<td>TGGCGATGGAGAAATGGCA</td>
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<tr>
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<td>GGAATTTGCTGGTGAGCTTCT</td>
<td>XM_346065</td>
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<tr>
<td>HDAC1</td>
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<td>TGCAATATACTTTAGAGCCGA</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>McP2</td>
<td>CGCGAAGCTTAAACAGAGGA</td>
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<tr>
<td>Probe:</td>
<td>6FAM-TACATCATACTTCCAGCACGAGCCACAC-TAMRA</td>
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DNMT, DNA methyltransferase; HDAC, histone deacetylase.
RESULTS

d0 and d21 cerebral DNA methylation. At d0, uteroplacental insufficiency significantly decreased genome-wide DNA methylation and CpG island methylation to 52.8 ± 11.3% and 65.0 ± 7.4% of control values, respectively (Fig. 1A; P < 0.05). As expected, no difference was noted in the MspI digests. No sex-specific differences were noted at d0 in terms of DNA methylation.

In contrast, at d21, sex-specific differences were noted. For d21 female rats, uteroplacental insufficiency and subsequent IUGR resulted in a persistence of CpG island hypomethylation to 55 ± 10 of control values (Fig. 1B; P < 0.01). No differences were noted in global DNA methylation between female Con and IUGR rats. For d21 male rats, no significant differences were noted in either global or CpG island methylation (Fig. 1C).

d0 and d21 cerebral histone acetylation. In the d0 IUGR rat brain, total histone H3 acetylation was increased to 157 ± 16.1% of control values (P < 0.05), whereas no difference was noted in total H4 histone acetylation. Site-specific changes were also evident; uteroplacental insufficiency significantly increased whole brain histone acetylation at H3/K14 to 188 ± 24 of control values (P < 0.05), without affecting H3/K9 acetylation (129 ± 27% of control values) (Fig. 2A). Interestingly, immunohistochemistry revealed that that H3/K14 acetylation (Fig. 3A) and H3/K9 acetylation (Fig. 4A) were increased in d0 white matter and hippocampus, respectively, in the IUGR rats relative to control. No sex-specific differences were noted at d0 in terms of histone acetylation.

In d21 female IUGR rats, total brain H3/K14 acetylation persisted to 123 ± 5.6% of control values (*P < 0.05; Fig. 2B). This persistence did not hold true for total brain H3/K9 acetylation, which was not significantly different between d21 IUGR and Con female rats. However, localized changes in H3/K14 and H3/K9 acetylation persisted in the d21 female rats, because immunohistochemistry demonstrated increased

Fig. 1. Levels of global and CpG island DNA methylation from control (C) and intrauterine growth retardation (IUGR; I) whole brains. A: day of life 0 (d0). B: day of life 21 (d21) female. C: d21 male. Results are expressed as mean ± SE % relative to sham-operated controls (n = 6 litters). *P < 0.05, **P < 0.01.
H3/K14 acetylation in the white matter and increased H3/K9 acetylation in the hippocampus, respectively (Figs. 3B and 4B).

In contrast, in d21 male rats, total brain H3/K14 acetylation was not significantly different between IUGR and Con animals, whereas H3/K9 acetylation was significantly reduced in brain from IUGR male rats to 68.3\%/110061.9 of control values (P < 0.001; Fig. 2C). Moreover, decreased acetylated H3/K14 and H3/K9 immunostaining were evident in the internal capsule and hippocampus of the male IUGR rats, respectively (Figs. 3C and 4C).

d0 Transmission electron microscopy. The ultrastructural appearance of nuclei of neurons in the hippocampus was dependent on the rat group. Nuclei appeared smaller with more condensed chromatin around the nuclear envelope in the brains from IUGR pups compared with the Con group, whereas the larger nuclei in the Con group had an open-faced vesicular profile (Fig. 5). Image analysis supported the observed difference in size and showed that neuronal nuclei were significantly smaller in the hippocampus from the IUGR pups (Table 2).

d0 Cerebral MeCP2 and DNMT1 gene expression. Uteroplacental insufficiency decreased DNMT1 mRNA and protein levels in IUGR pup brains to 50 ± 8.0% (P < 0.01) and 71.5 ± 8.0% (P < 0.05) of control values, respectively (Fig. 6A). Similarly, cerebral mRNA levels of MeCP2 in IUGR pups were decreased to 49.6 ± 8% (P < 0.01) of control values (Fig. 6B). Likewise, d0 cerebral protein levels of MeCP2 in IUGR pups were also decreased to 62 ± 7.8% of control values (P < 0.01; Fig. 6B). Immunohistochemistry revealed decreased expression of both DNMT1 and MeCP2 within the hippocampus of the IUGR pups relative to controls (Fig. 6, A and B). No sex-specific differences were noted at d0 for either DNMT1 or MeCP2.

d0 Cerebral HDAC1 gene expression and HDAC activity. Although d0 HDAC1 mRNA levels were not affected, uteroplacental insufficiency significantly decreased HDAC1 protein levels in d0 IUGR brains to 68.3 ± 13.5 of control values (P < 0.05; Fig. 6C). In association with this change, IUGR brain HDAC nuclear activity decreased to 75 ± 7.9% of control values (P < 0.05; Fig. 6C). No sex-specific differences were noted at d0 for these measures of HDAC expression and activity. As with MeCP2 and DNMT1, differences in HDAC1 protein expression between d0 IUGR and Con brains localized to the hippocampus, where decreased HDAC1 staining was observed in IUGR rats (Fig. 6C).

d0 Cerebral mineral concentrations and one-carbon metabolites. Uteroplacental insufficiency also significantly increased whole brain levels of zinc, while decreasing whole brain levels of iron (Table 3). Copper levels were not significantly affected by uteroplacental insufficiency. Uteroplacental insufficiency significantly increased cerebral levels of adenosine, cysteinyl-glycine, and cysteine in the IUGR pups relative to the sham-operated controls (Table 3). No significant differences were noted in homocysteine, methionine, SAM, and SAH between the two groups.

d21 Breast milk content. Breast milk from dams that underwent the IUGR surgery did not significantly differ from Con breast milk in terms of caloric, fat, and protein
content (Table 4). Furthermore, no differences existed between Con and IUGR breast milk in terms of zinc and sodium content (Table 4). Interestingly, increased iron content characterized the breast milk from the IUGR dams vs. breast milk from the Con dams (36.3 ± 5.3 vs. 26.5 ± 7.1 μg/dl; P < 0.05).

**DISCUSSION**

Uteroplacental insufficiency causes IUGR, complicates ~6% of all pregnancies, and impacts human health because of morbidities such as neurodevelopmental delay and neuroendocrine reprogramming (13, 27, 32, 102, 103). These long-term morbidities suggest epigenetics as a responsible mechanism. Epigenetics involves changing determinants of chromatin structure and allows adaptation to the deprived environment through gene expression via an initial investment of energy. The latter is true because DNA methylation and histone acetylation can be maintained through cell division and therefore encode heritable information (45, 95). However, little has been presented identifying specific molecular epigenetic changes in the brain of animals suffering a prenatal insult. We therefore focused this study on the IUGR brain. The novel findings from this work include 1) IUGR affects DNA methylation and histone acetylation; 2) epigenetic alterations occur through d21 and show sex specificity; 3) the effects of IUGR on cerebral epigenetic determinants are region specific; 4) a possible trigger for these changes includes cerebral zinc levels; and 5) at d21, caloric, protein, and fat breast milk content do not differ between Con and IUGR dams, although iron content is significantly increased in IUGR breast milk.

Our findings of H3 hyperacetylation and DNA hypomethylation in IUGR rat brain at d0 demonstrate that IUGR impacts epigenetic determinants of cerebral chromatin structure, which is visible in the hippocampus by transmission electron microscopy. To understand the process through which these changes occur, we initially targeted DNMT1, as opposed to other DNA methyltransferases, because DNMT1 represents the majority of methyltransferase activity in embryo lysates (62, 107). The
association between decreased cerebral DNMT1 mRNA and protein expression suggests that DNMT1 plays a role in IUGR hypomethylation.

Similarly, global cerebral DNA hypomethylation characterizes transgenic animals in which DNMT1 is knocked out in neuroblasts (25). In these transgenic animals, cerebral cells affected by DNA hypomethylation were lost within the first 3 wk of life, and global DNA methylation normalized relative to the wild-type animals. Our postnatal findings that global methylation was similar between d21 Con and IUGR pups also suggest a loss of postnatal hypomethylated cells. A notable difference between the transgenic study and the present study is that we assessed CpG island methylation and found that CpG hypomethylation persisted in the female IUGR rats at d21. These latter data support the concept that separate mechanisms regulate global DNA methylation and CpG methylation (45). Interestingly, adult mice with decreased levels of DNMT1 expression resist mild to moderate cerebral ischemia (22, 23). These findings raise the intriguing possibility that the decrease in fetal DNMT1 expression in the IUGR brain may be a protective response to minimize the impact of uteroplacental insufficiency on the central nervous system.

The decreased levels of MeCP2 within the IUGR brain may also contribute to cerebral DNA hypomethylation. MeCP2 is required to maintain CpG status of genomic DNA (44, 61). As a result, the appearance of decreased MeCP2 in the hippocampus of the IUGR brain is consistent with the notion that DNA from this region is relatively hypomethylated.

This notion is further supported by the finding of decreased HDAC1 protein in the IUGR fetal hippocampus because DNA hypomethylation associates with histone hyperacetylation, which occurs if HDAC activity is reduced. We focused on HDAC1 because it complexes with DNMT1 and in vitro studies demonstrate that hypoxia decreases HDAC1 protein levels (30, 38). Other investigators have found that multiple stimuli affect hippocampal neuronal histone acetylation. Levinson et al. (60) found that activation of N-methyl-D-aspartate receptors increased hippocampal H3 acetylation, although their studies did not identify the lysines that were specifically affected. Crosio et al. (15) demonstrated that kainic acid (a

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**Fig. 4.** Representative immunohistochemistry of H3/K9 (brown staining) from hippocampal region of control and IUGR brains of d0 pups (A), d21 females (B), and d21 males (C) (n = 6 litters).
glutamate receptor agonist) increased H3/K14 acetylation. We found increased acetylation of both H3/K9 and H3/K14 in the d0 IUGR and d21 female hippocampus and white matter, respectively.

Several studies provide insight into the meaning of these findings. In yeast, different cellular stresses induced specific histone acetylation patterns that led to expression of genes from similar functional classes (46). This referenced study found that acetylation of H3/K9 associated with intergenic regions of expressed genes and acetylation of H3/K9 and H3/K14 associated with open reading frames of expressed genes (46). Similarly, a large-scale study of histone modification patterns in human and mouse cells for chromosomes 21 and 22 found that 58% of the acetylated H3/K9 and H3/K14 sites coincided within 1 kb of the transcription start of a known gene (5). These studies identify histone acetylation as a mechanism through which uteroplacental insufficiency affects chromatin structure near transcription start sites.

Our findings of altered H3/K14 and H3/K9 acetylation in white matter and hippocampus, respectively, are consistent with these regions being vulnerable to perinatal insults. In rats, uteroplacental insufficiency decreases hippocampal weight and neurogenesis as well as decreasing gliogenesis in the white matter of the cerebellum (11). In humans, placental abnormalities such as infarction and intrauterine hypoxia have been associated with white matter injury (8, 31). Furthermore, voxel-based morphometry has been used to demonstrate that perinatal asphyxia is also associated with hippocampal atrophy in “healthy adolescents” (67).

The vulnerability of the hippocampus to injury is particularly interesting in light of its function and the adult morbidities associated with IUGR. The function of the hippocampus is to “sense” soluble molecules in the blood to perform feedback control, and evidence exists that the hippocampus modulates body physiology, including the hypothalamus-pituitary-adrenal (HPA) axis (26, 39, 59). A major morbidity of IUGR is the neuroreprogramming of the HPA axis. Fernald and Grantham-McGregor (27) found that IUGR in school-aged Jamaican children altered their stress response to psychological and physiological stimuli. Similarly, Cianfarani et al. (13) demonstrated that the neuroendocrine response is permanently altered in some IUGR children, which may affect their catch-up growth. Of particular interest in the latter study, IUGR children who did not catch up were characterized by increased serum levels of cortisol.

Little is known about how sex influences the cerebral epigenetics and subsequent gene expression response to the IUGR insult, although animal studies focusing on other tissues provide some insight. Studies utilizing rats rendered IUGR through uteroplacental insufficiency demonstrate sex-specific differences in growth, serum triglycerides, and gene expression (37, 52, 54). In liver, IUGR rats of both sexes showed decreased liver expression of CPTI at d21, but only the male IUGR rats showed decreased expression of CPTI at

Table 2. Size of hippocampal nuclei

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<th>IUGR</th>
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<td>Nuclear area, µm²</td>
<td>37.0±1.9</td>
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<td>Nuclear perimeter, µm</td>
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<td>Nuclear maximum diameter, µm</td>
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<td>Nuclear minimum diameter, µm</td>
<td>6.1±0.1</td>
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Values are means ± SD for n = 4 litters. IUGR, intrauterine growth retardation.
day 120 of postnatal life relative to the controls (52). Furthermore, at d21, the livers of male IUGR rats are characterized by a relative H3 hyperacetylation, whereas the livers of female IUGR rats are characterized by a relative H3 hypoacetylation. We speculate that these differences between tissues explain, at least in part, why the general IUGR phenotype is grossly similar between the sexes but many specific characteristics appear to differ.

To identify a possible cellular trigger of the IUGR cerebral epigenetic response, we measured cerebral zinc levels. Zinc deficiency is associated with IUGR, and zinc is a cofactor for DNMT1 and HDAC1 (4, 26, 41, 68, 69, 74, 99, 100). Further-

Fig. 6. A: quantification of real-time RT-PCR and Western blots of DNA methyltransferase 1 (DNMT1) species, as well as representative Western blots from d0 control and IUGR brain. Representative immunohistochemistry of DNMT1 (brown staining) is on right. B: quantification of real-time RT-PCR and Western blots of methyl-CpG binding protein 2 (MeCP2) as well as representative Western blots from d0 control and IUGR brain. Representative immunohistochemistry of MeCP2 (brown staining) from the hippocampal region of a d0 control brain is on right. C: quantification of real-time RT-PCR and Western blots of histone deacetylase (HDAC)1 and HDAC activity, as well as representative Western blots from d0 control and IUGR brain. Representative immunohistochemistry of HDAC1 (brown staining) from the hippocampal region of a d0 IUGR brain is on right. For A–C, protein was quantified with NIH Image software. Results are expressed as mean ± SE % relative to sham-operated controls (n = 6 litters). *P < 0.05, **P < 0.01.
more, zinc deficiency markedly alters histone solubility compared with other iron deficiencies (24, 70). In our model, IUGR significantly increased cerebral zinc levels, which is likely caused by the rise in metallothionein levels that other investigators have observed in rodent brains stressed by ischemia (93, 101, 106).

We also measured cerebral levels of the important trace elements iron and copper. Considering the association between IUGR and neurodevelopmental delay, our findings of decreased cerebral iron in the IUGR brain are relevant because of the link between decreased hippocampal iron and function in infants of diabetic mothers (75, 80, 87). Unfortunately, little is known about the effects of IUGR on cerebral iron concentrations. Georgieff et al. (34) suggest that live born infants who suffer from restricted maternal-fetal blood flow may be at significant risk for postnatal iron deficiency in multiple tissues, including brain. Moreover, a study from this group found that fetal iron deficiency increased the vulnerability of the rat hippocampus to hypoxic ischemic insult (84).

To investigate another possible cellular mechanism through which uteroplacental insufficiency alters epigenetic determinants of chromatin structure, we measured cerebral levels of metabolites involved in one-carbon metabolism. The most relevant of these metabolites are SAH and SAM, which play a role in the regulation of genome-wide DNA methylation (86). In liver, IUGR significantly increases SAH, a response previously demonstrated to be associated with DNA hypomethylation (9, 66). The subsequent failure of IUGR to affect cerebral SAH levels in association with DNA hypomethylation highlights the tissue-specific nature of the IUGR response. This tissue-specific response is not surprising in light of previous work that demonstrated that folate/methyl donor deficiency causes DNA hypomethylation and decreased DNMT activity in liver but not in other tissues (83).

Interestingly, although cerebral SAH levels were not affected, uteroplacental insufficiency did increase cerebral cysteine, cysteinylglycine, and adenosine levels, all by-products of one-carbon metabolism. Cysteine is the rate-limiting precursor for glutathione synthesis (6). Astrocytes provide cysteine to neurons by releasing cysteinylglycine (105). Adenosine is released from cells under oxidative stress and acts as a neuroprotectant in the rodent hippocampus by attenuating the cellular consequences of reactive oxygen species (1, 85). As a result, the increased levels of all three metabolites (cysteine, cysteinylglycine, adenosine) may reflect an attempt by the IUGR fetus to protect the brain from oxidative stress.

Early postnatal nutrition is important, and the d21 cerebral epigenetic response may also reflect differences in postnatal nutrition, which is why we characterized breast milk from Con and IUGR dams after 21 days of supporting their respective pups. No significant differences in calories, protein, fat, or zinc were noted between the two groups, although differences may have existed earlier. Furthermore, other components of the breast milk not assessed in this study may have contributed to the differences at d21 between the epigenetic characteristics of the Con and IUGR rat brains. The differences in iron content between Con and IUGR dam breast milk are intriguing, because iron content is independent of maternal mineral status and decreased iron levels characterize the IUGR brain (17). We chose not to cross-foster, because this rarely occurs in the human situation and is unlikely to be recommended in the future because of infection issues.

Despite our best efforts, this latter point emphasizes that caution is always necessary when attempting to apply data from a rat model to human pathophysiology. The fetal and juvenile rat is physiologically immature relative to the human, and the insult imposed on the fetal rat in this model of uteroplacental insufficiency is severe. In contrast, the impact of uteroplacental insufficiency experienced by humans ranges across a continuum.

In summary, uteroplacental insufficiency and subsequent IUGR affect epigenetic determinants of chromatin structure in regions of the fetal brain that are known to be vulnerable to perinatal insults such as hypoxia or ischemia. The effects are both sex- and tissue specific. We speculate that changes in epigenetic determinants of chromatin structure alter gene expression in the IUGR rat hippocampus and subsequently trigger the neuroendocrine reprogramming that complicates the IUGR phenotype.

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GRANTS

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