Intrauterine growth retardation affects expression and epigenetic characteristics of the rat hippocampal glucocorticoid receptor gene

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Ke X, Schober ME, McKnight RA, O’Grady S, Caprau D, Yu X, Callaway CW, Lane RH. Intrauterine growth retardation affects expression and epigenetic characteristics of the rat hippocampal glucocorticoid receptor gene. Physiol Genomics 42: 177–189, 2010. First published April 13, 2010; doi:10.1152/physiolgenomics.00201.2009.—Studies in humans and rats suggest that intrauterine growth retardation (IUGR) permanently resets the hypothalamic-pituitary-adrenal (HPA) axis. HPA axis reprogramming may involve persistently altered expression of the hippocampal glucocorticoid receptor (hpGR), an important regulator of HPA axis reactivity. Persistent alteration of gene expression, long after the inciting event, is thought to be mediated by epigenetic mechanisms that affect mRNA and mRNA variant expression. GR mRNA variants in both humans and rats include eleven 5’-end variants and Grα, the predominant 3’-end variant. The 3’-end variants associated with glucocorticoid resistance in humans (GRβ, GRγ, GRA, and GRP) have not been reported in rats. We hypothesized that in the rat hippocampus IUGR would decrease total GR mRNA, increase Grβ, Grγ, GRA, and GRP, and affect epigenetics of the GR gene at birth (D0) and at 21 days of life (D21). IUGR increased hpGR and exon 1.7 hpGR mRNA in males at D0 and D21, associated with increased trimethyl H3/K4 at exon 1.7 at both time points. IUGR also increased hpGrβ in males at D0 and D21, associated with increased acetyl H3/K9 at exon 3 at both time points. hpGrγ increased in female IUGR rats at D0 and D21. In addition, our data support the existence of hpGrβ and hpGrγ in the rat. IUGR has sex-specific, persistent effects on GR expression and its histone code. We speculate that postnatal changes in hippocampal GR variant and total mRNA expression may underlie IUGR-associated HPA axis reprogramming.

A key regulator of HPA axis reactivity is the hippocampal glucocorticoid receptor (hpGR) (25). Glucocorticoid binding to the hpGR initiates a negative feedback loop that terminates stress-induced HPA axis activation (12). In rodent models of IUGR induced by maternal undernutrition, hpGR expression was decreased at birth (41) or prenatally (43). If persistent, such alterations in hpGr gene expression could contribute to the long-lasting effects of IUGR on the HPA axis.

Changes in gene expression that remain long after the inciting conditions have resolved are often attributed to epigenetic phenomena (2, 15). Epigenetic phenomena are modifications to chromatin and/or to DNA that do not alter the sequence of DNA but rather its accessibility to the transcription machinery, thereby affecting gene expression (10, 50, 55). Examples of such epigenetic modifications include histone acetylation/methylation and DNA methylation. Using our model of IUGR, we have previously shown (19, 21) gene-specific epigenetic modifications associated with persistently altered gene expression.

Epigenetic regulation is classically associated with differences in mRNA variant expression. The GR gene produces multiple mRNA variants, broadly categorized as NH2-terminal (5’ end) or COOH-terminal (3’ end) variants (Fig. 1). 5’-End variants result in the same protein product. They differ from each other another in promoter region usage and transcription initiation sites within exon 1. Importantly, the 5’-end variants are tissue specific. While the hippocampus expresses all 11 exon 1 variants (66), this is not the case for other tissues studied. For example, exon 1.5 and 1.7 variants are highly abundant in the hippocampus (49) but are absent in liver and lung; both liver and lung express exon 1.11 variants (66). The four 3’-end variants (GRβ, GRγ, GRA, and GRP) described in humans result in different proteins and are associated with glucocorticoid resistance in childhood leukemia (6, 45) and in human myeloma (35, 46, 47, 53) (Supplemental Table S1).1

The GR gene is highly conserved between humans and rats. All eleven 5’-end variants described in the rat (49) have been found in the human (66), as shown in Fig. 1. These variants are derived from 7 human and 11 rat alternative exon 1s. Thus far only one of the known human 3’-end variants, GRα, has been described in the rat (58, 63).

Despite the importance of GR variants on GR expression and function, to our knowledge no studies have examined the effect of IUGR on GR mRNA variant expression. Furthermore, although uteroplacental insufficiency (UPI) is the predominant cause of IUGR in the developed world (40), the effect of UPI-induced IUGR on the expression or epigenetic modifici-
tions of GR has not been studied. Finally, to date, studies on GR mRNA variant expression in the rat have not addressed the possible existence of 3′-end variants other than GRα.

Our well-established model of UPI-induced IUGR, consisting of bilateral uterine artery ligation in the pregnant Sprague-Dawley dam at day 19 of gestation (19, 31), results in rat pups that are 20% lighter than control pups at birth and grow up to develop insulin resistance, obesity, and hypertension (5, 38, 76). Using our model, we found elevated circulating cortisol levels in the fetus (3, 4) and 21-day-old (D21) rat pup (3, 4). Similarly, in a model of IUGR induced by maternal undernutrition, fetal cortisol levels were confirmed by PCR and sequencing. Hippocampal mRNA levels of total GR and GR 5′- and 3′-end splice variants were measured by real-time RT-PCR at D0 and D21. In brief, CDNA was synthesized from 2 μg of RNA, and probe and primers were added to Taqman Universal PCR master mix (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. Hippocampi 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 protein inhibitors and 0.1 M PMSF, and supernatants were obtained by centrifugation. Fifty to one hundred micrograms of total protein was run on 10% SDS-PAGE (Bio-Rad, Hercules, CA) and transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% milk [3% BSA for phosphorylated GR (pGR)], bound proteins were exposed to specific antibodies to GR and GRα (ab3578 and ab3580, Abcam) and pGRser211 and GAPDH (4161 and 2118, Cell Signaling Technology, Beverly, MA) at 4°C overnight. Blots were detected with Western Lightening enhanced chemiluminescence (ECL) (PerkinElmer Life Sciences) and goat anti-rabbit horseradish peroxidase (HRP) secondary antibody (Cell Signaling Technology) and quantified on a Kodak Image Station 2000R (Eastman Kodak/SIS, Rochester, NY).

Immunohistochemistry. One coronal section per animal was taken at around bregma at 3.00 mm from D0 and D21 brains. After standard deparaffinizing and rehydrating steps, sections were subjected to antigen retrieval procedure (DakoCytomation, Carpinteria, CA). Slides were blocked (TSA Biotin System kit, PerkinElmer) and exposed to anti-GR antibody (ab3578, Abcam) overnight at 4°C. After washing, sections were exposed to appropriate secondary antibodies and stained with diaminobenzidine (DAB; Sigma, St. Louis, MO), counterstained with hematoxylin, dehydrated, and coverslipped.
DNA isolation and sodium bisulfite sequencing. Genomic DNA was extracted from hippocampi by overnight proteinase K digestion at 56°C (10 mM Tris, pH 7.6, 25 mM EDTA, 75 mM NaCl, 1% SDS, 180 μg/ml proteinase K) and centrifugation at 13,000 rpm. Supernatant was vortexed briefly with a saturated NaCl solution, and an equal volume of chloroform was added to each tube. Cold absolute ethanol was added in a 2-to-1 ratio to the supernatant and resup. The pellet was washed with graded series of ethanol, and the resultant DNA was dried and resuspended. One microliter of RNase (20 mg/ml) was added to each tube and incubated at 37°C for 30 min. DNA was quantified by standard spectrophotometry and subjected to sodium bisulfite modification according to the manufacturer’s protocol (CpGenome DNA modification Kit, Chemicon International, Temecula, CA) to determine site-specific CpG methylation. On the basis of our mRNA results, DNA from CpG-rich promoter regions of exons 1.6 and 1.7 (D0 and D21) and of exon 1.10 (D21) were selected for PCR amplification using outside primers, and the PCR product was used as a template for subsequent PCR reactions using inside primers. For exon 1.6 and 1.10, primers were designed for the regions 100–200 bp upstream of the transcription initiation site, while primers for exon 1.7 were based on previously published promoter regions for this variant (71, 72) (Supplemental Table S3). PCR conditions were 95°C for 10 min, followed by 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min for 35 cycles. The PCR products for exons 1.7, 1.6, and 1.10 (amplicon lengths of 177, 94, and 139 bp, respectively) were cloned into pCR2.1 with the TOPO TA Cloning Kit (Invitrogen, San Diego, CA). Multiple colonies were sequenced according to the manufacturer’s instructions for double-stranded plasmid DNA with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Chromatin immunoprecipitation assay and real-time PCR. Chromatin immunoprecipitation (ChIP) with anti-acetyl H3/K9, anti-acetyl H3/K14, anti-dimethyl H3/K4, anti-trimethyl H3/K4, anti-trimethyl H3/K9, and anti-trimethyl H3/K36 (Cell Signaling Technology) was performed as described previously (20). Real-time PCR was used to quantitate the amount of DNA from the GR promoter regions upstream of exons 1.5, 1.7, 1.11, 3, 4, 7, 9α, and 9β and an intergenic sequence 250 kb upstream of the insulin-like growth factor I (IGF-I) gene used as an internal control (Supplemental Table S4). We excluded the exon 1.6 promoter region from CHIP assay because its high CpG content precluded primer probe design.

Statistics. All data are expressed as mean ± SE percentages of control. We used ANOVA (Fisher’s protected least significant difference) and the Student’s unpaired t-test. Statistical significance was set at P < 0.05.

RESULTS

IUGR altered hpGR and variant mRNA levels in sex-specific manner. Contrary to expectations, IUGR increased GR total mRNA in male pups at both D0 (123 ± 5% of control) and D21 (154 ± 15% of control) (both P < 0.05; Fig. 2A). In females, total GR mRNA in IUGR females also increased at D21 (175 ± 13% of control; P < 0.01), but at D0 it was the same as control. IUGR also had sex-specific effects on GR mRNA exon 1 variant expression (Fig. 2B). Interestingly, exon 1.7 mRNA levels increased in IUGR males at both D0 and D21 (246 ± 42% and 182 ± 29% of control values, respectively; both P < 0.05) but did not change in females. In contrast, in female IUGR pups exon 1.11 mRNA levels decreased (27 ± 6% of control; P < 0.01) at D0, and exon 1.6 and exon 1.10 mRNA levels increased at D21 (158 ± 26% and 163 ± 17% of control; both P < 0.05) (Fig. 2B). We found no differences in exon 1.5 mRNA levels at any time. All negative results are shown in Supplemental Fig. S2b.

Our results support existence of GR 3′-end splicing variants in rat hippocampus. IUGR increased male rat GRγ mRNA (172 ± 13% of control; P < 0.05) and decreased GRP mRNA (31 ± 10% of control; P < 0.05) at D0. At D21, IUGR elevated mRNA levels of GRα (287 ± 83% of control; P < 0.05), GRγ (135 ± 7% of control; P < 0.01), and GRA (181 ± 16% of control; P < 0.01) and decreased mRNA levels of GRβ (56 ±
13% of control; \( P < 0.01 \) in males (Fig. 2C). In contrast, in D0 females, IUGR decreased GR\( \gamma \) mRNA (68 ± 5% of control; \( P < 0.05 \)), while it increased mRNA levels of GRA and GRP [155 ± 5% \( (P < 0.01) \) and 355 ± 112% \( (P < 0.05) \) of control values, respectively]. At D21, IUGR increased mRNA levels of GR\( \gamma \) (175 ± 15% of control; \( P < 0.001 \)) and GRA (242 ± 23% of control; \( P < 0.001 \)) and decreased mRNA levels of GR\( \alpha \) (54 ± 16% of control; \( P < 0.05 \)) in females (Fig. 2D).

All negative results are shown in Supplemental Fig. S2, c and d.

There are no commercially available antibodies for the 3′-end variants, except for GR\( \beta \) (in humans) and GR/GR\( \alpha \) (both rats and humans). To our knowledge, no published studies to date report on other 3′-end variant protein levels, likely because the predicted COOH-terminal proteins for GR\( \alpha \), \( \beta \), \( \gamma \), and P are very similar, differing by as little as one amino acid within the DNA-binding domain (39).

IUGR increased levels of hippocampal GR and phosphorylated GR and GR\( \alpha \) protein levels. hpGR protein in male IUGR did not change at D0 but increased at D21 (230 ± 47% of control; \( P < 0.05 \)), consistent with mRNA results (Fig. 3A). In IUGR females, hpGR protein levels were similarly unchanged at D0 and elevated at D21 (295 ± 35% of control values; \( P < 0.001 \)) (Fig. 3B). IUGR increased hippocampal pGRser211 (pGR) protein levels in males at D0 (157 ± 20% of control; \( P < 0.05 \)) and D21 (204 ± 42% of control; \( P < 0.05 \)) (Fig. 3C).
in IUGR females they increased only at D21 (170 ± 6% of control; 
$P < 0.01$) (Fig. 3D). GR$\alpha$ protein levels increased in IUGR male 
hippocampus at D0 and D21 [164 ± 4% ($P < 0.0001$) and 207 ± 
22% ($P < 0.01$) of control] (Fig. 3E), while GR$\alpha$ protein levels did 
not change in females at any time (Fig. 3F).

Our qualitative immunohistochemistry (IHC) studies suggested 
that, for both sexes, differences in GR expression were not confined 
to a particular hippocampal region at D0 (Fig. 4) or at D21 (Fig. 5).

IUGR did not alter hippocampal DNA methylation patterns in 
promoter regions of GR at exons 1.6, 1.7, and 1.10. Bisulfite 
sequencing did not show differences in DNA methylation on the GR 
exon 1.6 and 1.7 promoters in IUGR hippocampus at D0 or on the 
GR exon 1.6, 1.7, or 1.10 promoters at D21 (Supplemental Fig. S1).

IUGR had both sex- and time-specific effects on histone code 
at GR promoter regions upstream of exon 1 variants. In males, 
IUGR increased trimethyl H3/K4 at exon 1.7 at both D0 and D21.

Fig. 4. Representative immunohistochemistry of GR (brown staining) for hippocampus of D0 control male pups (A), IUGR male pups (B), control female pups (C), and IUGR female pups (D), $n = 4$ rats from different litters per group. Scale bar, 100 μm.

Fig. 5. Representative immunohistochemistry of GR (brown staining) for hippocampus of D21 control male pups (A), IUGR male pups (B), control female pups (C), and IUGR female pups (D). Granular staining of GR in boxed areas is shown in insets. Arrows show representative glucocorticoid receptor-positive cells. $n = 4$ rats from different litters per group. Scale bar, 100 μm.
For exon 1.5, IUGR increased dimethyl H3/K4 (158 ± 15% of control; P < 0.05) at D0 and increased trimethyl H3/K36 (197 ± 19% of control; P < 0.01) at D21 (Fig. 6A). For exon 1.7, IUGR increased dimethyl H3/K4 and trimethyl H3/K4 and K36 [184 ± 19% (P < 0.01), 130 ± 9% (P < 0.05), and 194 ± 37% (P < 0.05) of control, respectively] at D0, with increased trimethyl H3/K4 [281 ± 65% of control (P < 0.05)] persisting into D21. Finally, trimethyl H3/K9 was decreased (20 ± 3% of control; P < 0.05) in IUGR pups at D21 (Fig. 6B). For exon 1.11, IUGR elevated dimethyl H3/K4 (173 ± 24% of control; P < 0.05) at D0 but decreased dimethyl H3/K4 and trimethyl H3/K9 (54 ± 15% and 23 ± 5% of control; both P < 0.05) at D21 (Fig. 6C). We found no differences in the histone code upstream of exon 1.6 or 1.10 for males at any time. All negative results are shown in Supplemental Fig. S6, a–c.

In females, IUGR increased trimethyl H3/K9 at exon 1.7 and decreased acetyl H3/K14 at exon 1.5 at both D0 and D21. For exon 1.5, IUGR decreased acetyl H3/K14 at both D0 and D21 (75 ± 3% and 74 ± 7% of control; both P < 0.05). IUGR also decreased acetyl H3/K9 and trimethyl H3/K36 [41 ± 1.3% (P < 0.01) and 61 ± 7% (P < 0.05) of control, respectively] at D21 (Fig. 6D). For exon 1.7, acetyl H3/K9 and trimethyl H3/K9 (244 ± 36% and

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Fig. 6. Histone markers associated with GR exon 1 splicing variants at D0 and D21, expressed as % of sex-matched control ± SE. Only data significantly different between groups are shown, while negative results are listed in RESULTS and Supplemental Fig. S6, a–f. CM, Control male; CF, Control female; IM, IUGR male; IF, IUGR female. n = 4 (D21) and n = 4 (10 pooled D0 hippocampi per sample) rats from different litters. Graphs represent histone markers associated with promoter regions of exon 1.5 (males, A; females, D), exon 1.7 (males, B; females, E) and exon 1.11 (males, C; females, F). *P < 0.05, **P < 0.01.
242 ± 58% of control, respectively; both \( P < 0.05 \) were elevated in IUGR pups at D0, with increased trimethyl H3/K9 (349 ± 92% of control; \( P < 0.05 \)) persisting into D21 (Fig. 6E). For exon 1.11, increased acetyl H3/K9 and decreased dimethyl H3/K4 (212 ± 41% and 90 ± 2% of control, respectively; both \( P < 0.05 \)) were found in female IUGR pups at D0, while at D21 trimethyl H3/K9 increased (264 ± 56% of control; \( P < 0.05 \)) (Fig. 6F). We found no differences in the histone code upstream of exon 1.6 or 1.10 for females at any time. All negative results are shown in Supplemental Fig. S6, d–f.

IUGR had both sex- and time-specific effects on GR histone code at 3' = end variants, with acetyl H3/K9 consistently affected at exon 3 and dimethyl H3/K4 consistently affected at exon 9β in males. In males, IUGR increased acetyl H3/K9 but decreased acetyl H3/K14 (217 ± 28% (\( P < 0.01 \)) and 55 ± 8% (\( P < 0.05 \)) of control) on exon 3 at D0. At D21, IUGR increased acetyl H3/K9, K14, and dimethyl H3/K4 (160 ± 4% (\( P < 0.05 \)), 131 ± 9% (\( P < 0.05 \)), and 136 ± 4% (\( P < 0.05 \)) of control, respectively) (Fig. 7A). For exon 4, IUGR did not change the histone code at D0 but increased dimethyl H3/K4 (138 ± 8% of control; \( P < 0.05 \)) at D21 (Fig. 7B). On exon 7, IUGR increased acetyl H3/K9 (127 ± 5% of control; \( P < 0.05 \)) at D0 and increased dimethyl H3/K4 and trimethyl H3/K9 (141 ± 10% and 245 ± 69% of control, respectively; both \( P < 0.05 \)) at D21 (Fig. 7C). For exon 9α, IUGR increased trimethyl H3/K9 (165 ± 23% of control; \( P < 0.05 \)) at D0 but not at D21 (Fig. 7D). For exon 9β, IUGR decreased dimethyl H3/K4 at both D0 (35 ± 11% of control; \( P < 0.05 \)) and D21 (32 ± 7% of control; \( P < 0.05 \)) and increased acetyl H3/K9 (380 ± 128% of control; \( P < 0.05 \)) levels at D21 only (Fig. 7E). All negative results are shown in Supplemental Fig. S7.

IUGR increased dimethyl H3/K4 at exon 7 in females at both time points. In IUGR females at exon 3, dimethyl H3/K4 increased (169 ± 23%* of control; \( P < 0.05 \)) at D0 while trimethyl H3/K4 decreased (38 ± 8% of control; \( P < 0.05 \)) at D21 (Fig. 8A). For exon 4, acetyl H3/K14 decreased (68 ± 4% of control; \( P < 0.05 \)) at D0 and trimethyl H3/K9 decreased (61 ± 0.6% of control; \( P < 0.05 \)) at D21 (Fig. 8B). For exon 7, dimethyl H3/K4 and trimethyl H3/K4 increased (136 ± 12% and 118 ± 4% of control, respectively; both \( P < 0.05 \)) at D0, with increased dimethyl H3/K4 (193 ± 40% of control; \( P < 0.05 \)) persisting into D21. Trimethyl H3/K36 (185 ± 22% of control; \( P < 0.05 \)) decreased dimethyl H3/K4 at exon 7 in females at both time points. In IUGR females at exon 3, dimethyl H3/K4 increased (169 ± 23%* of control; \( P < 0.05 \)) at D0 while trimethyl H3/K4 decreased (38 ± 8% of control; \( P < 0.05 \)) at D21 (Fig. 8A). For exon 4, acetyl H3/K14 decreased (68 ± 4% of control; \( P < 0.05 \)) at D0 and trimethyl H3/K9 decreased (61 ± 0.6% of control; \( P < 0.05 \)) at D21 (Fig. 8B). For exon 7, dimethyl H3/K4 and trimethyl H3/K4 increased (136 ± 12% and 118 ± 4% of control, respectively; both \( P < 0.05 \)) at D0, with increased dimethyl H3/K4 (193 ± 40% of control; \( P < 0.05 \)) persisting into D21. Trimethyl H3/K36 (185 ± 22% of control; \( P < 0.05 \))
also increased in IUGR pups at D21 (Fig. 8C). On exon 9α, IUGR decreased acetyl H3/K9 but increased dimethyl H3/K4 and trimethyl H3/K9 [30 ± 6% (P < 0.001), 268 ± 61% (P < 0.05), and 327 ± 48% (P < 0.01) of control, respectively] at D0. At D21, IUGR increased trimethyl H3/K4 (387 ± 128% of control; P < 0.05) levels (Fig. 8D). On exon 9β, dimethyl H3/K4 and trimethyl H3/K4 increased (146 ± 20% and 151 ± 22% of control; P < 0.05) at D0, with no histone changes at D21 (Fig. 8E). All negative results are shown in Supplemental Fig. S8.

**DISCUSSION**

The most important findings in this work are that 1) UPI-induced IUGR increased hpGR mRNA and phosphorylated hpGR protein at birth in males and increased hpGR mRNA, protein, and phosphorylated hpGR protein at D21 of life in both sexes, 2) UPI-induced IUGR was associated with sex-specific changes in hpGR mRNA variants and histone modifications that persisted through D21, and 3) our sequencing results support the hypothesis that the 3′-end mRNA variants GRβ, GRγ, GRA, and GRP exist in the rat hippocampus and are altered by UPI-induced IUGR.

Our findings of increased hpGR are very similar to studies of IUGR induced by a maternal low-protein diet in the rat. These investigators found increased GR expression in the IUGR brain throughout the lifespan in association with persistent hypertension (7) as well as decreased kidney 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2). Interestingly, we have previously demonstrated (5) decreased kidney 11β-HSD2 at D0 and D21 in our model as well. Similar to our model, hpGR expression was also increased in a sheep model of postnatal hypertension induced by prenatal glucocorticoid exposure (14).

Our results contrast in some respects with studies in IUGR models using rodent undernutrition (caloric restriction). Newborn rats exposed to maternal caloric restriction had decreased hpGR expression at birth (41, 68) but by D21, similar to our findings, had increased hpGR expression (68). In a different model, hpGR expression decreased in the fetal guinea pig after very brief maternal caloric restriction (43), but hpGR expression in later life was not studied.

We found pGRser211 protein levels in males at D0 and in both sexes at D21 in the IUGR hippocampus, suggesting that UPI-induced IUGR may increase hpGR transcriptional activity.
In light of our previous findings that IUGR increased circulating corticosterone in D0 and D21 rat pups (3), elevated GR mRNA/protein and pGR expression in the face of elevated corticosterone levels is contrary to our expectations, yet not unprecedented. While in most adult cells and tissues GR mRNA levels are negatively regulated by increased circulating corticosteroids (29, 64), developing animals may not respond in the same manner. Adult rats exposed to prenatal stress had HPA axis hyperreactivity, increased circulating corticosterone levels, and increased hippocampal GR expression (65). In addition, Wei et al. (74) demonstrated that transgenic mice with GR overexpression in forebrain display normal basal circulating corticosterone levels and, paradoxically, have a delayed termination of the stress response. A final point is that, of course, regulation of GR expression is highly complex and involves many factors other than cortisol, such as vasopressin and corticotropin-releasing hormone (24).

In male IUGR rats, we found that increased hpGR mRNA expression at ages D0 and D21 was associated with increased exon 1.7 mRNA variant expression at both time points. Similarly, Weaver et al. (73) showed that increased total hpGR mRNA expression was associated with increased exon 1.7 mRNA variant expression in a study of postnatal differential maternal care that examined only male rat offspring. We also found epigenetic changes associated with this persistent change in gene expression, albeit of a different nature. While we did not find hypomethylation in the GR promoter region as noted by Weaver et al., we did find persistent changes in histone modifications associated with GR mRNA and exon 1.7 mRNA upregulation in IUGR males. It is possible that the timing and nature of the perinatal insult plays a large role in these differences.

We found that UPI-induced IUGR had sex-specific effects on the expression and epigenetic characteristics of GR and its variants. Sex specificity has been noted numerous times with early life insults. For example, maternal separation induced more severe hippocampal neuronal degeneration in male than female rats (44). Also, IUGR has sex-specific effects in humans and in rats. In humans, there are marked sex differences in the relationship between birth weight and the stress response (60). Rats rendered IUGR through UPI demonstrate sex-specific differences in growth, serum triglycerides, and gene expression (23, 36, 37). We speculate that the epigenetic differences we found in our study may underlie, at least in part, the sex-specific effects of IUGR on the adult phenotype in our model.

![Fig. 9. Summary of exon 1, 3'-end mRNA variants, and associated histone modifications found in IUGR males, relative to control males, at D0 and D21. The GR gene cartoon illustrates schematically the locations (represented as circles) used for chromatin immunoprecipitation (ChIP) assay specific to each mRNA variant. Increased, decreased, or unchanged values for mRNA, protein, and histone modifications relative to control are represented by up, down, or horizontal arrows, respectively. For simplicity, unchanged values for histone modifications are represented by blank spaces only.](http://physiolgenomics.physiology.org/FIG_9.jpg)

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We found expected histone modifications in males, but not in females, associated with changes in gene expression that persisted at both D0 and D21. In general, acetylation of histones is correlated with transcriptional activation. Methylation of histones, on the other hand, is associated with either transcriptional activation (H3/K4, H3/K36, H3/K79) or transcriptional repression (H3/K9 and H3/K27). Accordingly, we found that increased trimethyl H3/K4 at exon 1.7 at both D0 and D21 males was associated with increased exon 1.7 mRNA and GR mRNA levels at these time points, as were increased trimethyl H3/K36 and dimethyl H3/K4 at D0 and decreased trimethyl H3/K9 at D21, respectively. Similarly, in males, we found increased acetyl H3/K9 at D0 and D21 as well as increased dimethyl H3/K4 at D21 at exon 3 associated with persistently increased GRα mRNA at D0 and D21. While in females GRA mRNA was persistently increased at D0 and D21, however, we only found decreased trimethyl H3/K9 at exon 4 at D21 and not at D0. Figures 9 and 10 summarize changes in histone modifications found in IUGR rats at D0 and D21, as well as the associated alterations in variant mRNA expression.

The histone code is undoubtedly complex. What is becoming clear is that persistent changes in gene transcription often depend not on a single epigenetic modification but upon the interactions between multiple alterations (67) and on variables such as nucleosome positioning (11). Multiple interdependent or independent histone modifications affect gene transcription (13, 34). Moreover, multiple signaling pathways converge upon histones (9). The complexity of the histone code is underlined by studies in which trimethyl H3/K4 and K36 have been associated with gene repression (8, 16, 27, 32, 33, 54).

In humans, changes in 3′-end GR mRNA variant expression have been linked to functional consequences. Given the remarkable conservation between species, it is possible that rat 3′-end GR mRNA variants behave similarly to human variants, but we cannot derive any conclusions regarding their functional significance. In humans, glucocorticoid resistance correlates with the expression of GRP, GRβ, GRγ, and GRA (6, 53). If this were true in the rat as well, then our finding of increased GRα and decreased GRβ expression, together with increased GR in the IUGR male hippocampus, would suggest increased hpGR activity. We also speculate that the persistently increased GRA in females, and increased GRγ in males, could be a compensatory mechanism to decrease GR responsivity in the hippocampus.

Fig. 10. Summary of exon 1, 3′-end mRNA variants, and associated histone modifications found in IUGR females, relative to control females, at D0 and D21. The GR gene cartoon illustrates schematically the locations (represented as circles) used for ChIP assay specific to each mRNA variant. Increased, decreased, or unchanged values for mRNA, protein, and histone modifications relative to control are represented by up, down, or horizontal arrows, respectively. For simplicity, unchanged values for histone modifications are represented by blank spaces only.
The limitations of our study are that we did not study dissected hippocampal subfields but rather used whole hippocampus for quantitative immunoblotting. Nevertheless, our IHC data support uniform effects across the various hippocampal areas. In addition, we used homogenized whole hippocampus for each group, thus minimizing the effects of any one cell population within a study group. Finally, two studies suggest that GR expression across hippocampal subfields may be similar; in a rat model of restraint stress, GR expression was robustly decreased equally in all subfields (56), while in a model of rat starvation GR was unaffected in all subfields (48).

The data beg two questions that are beyond the scope of this article. First, what are the mechanisms underlying changes in the GR epigenetic profile in IUGR rats? Because we found minimal differences in DNA methylation, we speculate that one potential mechanism is IUGR-associated nucleosome repositioning, such as that involving the ATP-sensitive SWI/SNF complex. Second, what are the physiological consequences of altered GR expression in IUGR animals? We speculate that UPI-induced IUGR rats would have decreased HPA axis reactivity to an acute stressor, given that increased hippocampal GR mRNA expression is associated with decreased HPA axis reactivity in male Lewis rats (26), outbred rats exposed to neonatal handling (51, 52), and GR-overexpressing transgenic mice (74). Interestingly, GR overexpression is also associated with impaired cognitive function (74), and blocking GR blunts the detrimental effects of high circulating corticosteroids on neurogenesis and memory. Both questions are important and clearly deserve further study.

In summary, our data support the existence of the hereto unreported GR 3'-end mRNA variants GRβ, GRy, GRA, and GRP in rat hippocampus. Further studies are needed to delineate the functional significance of this finding. UPI and subsequent IUGR increased variant and total mRNA expression of the GR gene, as well as total GR and pGR protein levels, in the D21 rat hippocampus, similar to studies of IUGR induced by maternal low-protein diet and in models of perinatal stress. We also found sex-specific alterations in total GR mRNA and variant mRNA variant expression and histone code that persist to D21. The GR gene is highly complex, and hence the effects of any insult on its expression must take the existence of GR mRNA variants and epigenetics into account. We speculate that these changes in the GR histone code may affect subsequent postnatal GR expression and signaling, which in turn may affect programming of the HPA axis at adulthood.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


