Prenatal transportation stress alters genome-wide DNA methylation in suckling Brahman bull calves^{1,2}

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ABSTRACT: The objective of this experiment was to identify genome-wide differential methylation of DNA in young prenatally stressed (PNS) bull calves. Mature Brahman cows ($n = 48$) were transported for 2-h periods at 60 ± 5 , 80 ± 5 , 100 ± 5 , 120 ± 5 , and 140 ± 5 d of gestation or maintained as nontransported Controls ($n = 48$). Methylation of DNA from white blood cells from a subset of 28-d-old intact male offspring ($n = 7$ PNS; $n = 7$ Control) was assessed via reduced representation bisulfite sequencing. Samples from PNS bulls contained 16,128 CG, 226 CHG, and 391 CHH (C = cytosine; G = guanine; H = either adenine, thymine, or cytosine) sites that were differentially methylated compared to samples from Controls. Of the CG sites, 7,407 were hypermethylated (at least 10% more methylated than Controls; $P \leq$

0.05) and 8,721 were hypomethylated (at least 10% less methylated than Controls; $P \leq 0.05$). Increased DNA methylation in gene promoter regions typically results in decreased transcriptional activity of the region. Therefore, differentially methylated CG sites located within promoter regions ($n = 1,205$) were used to predict (using Ingenuity Pathway Analysis software) alterations to canonical pathways in PNS compared with Control bull calves. In PNS bull calves, 113 pathways were altered ($P \leq 0.05$) compared to Controls. Among these were pathways related to behavior, stress response, metabolism, immune function, and cell signaling. Genome-wide differential DNA methylation and predicted alterations to pathways in PNS compared with Control bull calves suggest epigenetic programming of biological systems in utero.

Key words: *Bos indicus*, calves, DNA methylation, epigenetics, prenatal stress, reduced representation bisulfite sequencing

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INTRODUCTION

Environmental stimuli or stressors incurred by a gestating dam can alter postnatal phenotypes of the offspring (Clarke et al., 1996; Lay et al., 1997; Mueller and Bale, 2008). In response to a stressor experienced by a gestating female, the placental barrier enzyme 11- β HSD2 might be suppressed. Because 11- β HSD2 functions to convert cortisol to an inactive form before crossing the placenta to fetal circulation, decreased 11- β HSD2 results in increased exposure of the fetus to corticosteroids (Benediktsson et al., 1997; Stirrat et al., 2018). Altered prenatal environment affects postnatal outcomes, in part, by epigenetic modifications, such as DNA methylation (Szyf, 2012). DNA methylation is a covalent modification in which a methyl group is added to the carbon 5 position of a cytosine nucleotide (Wyatt, 1950; Razin and Riggs et al., 1980). This chemical modification of DNA plays a crucial role in regulating unique functions between different cell types, despite each cell type having an identical genome (Razin and Riggs, 1980; Suelves et al., 2016). It is not well understood how a prenatal or life experience might alter DNA methylation differently between cell types, though there is evidence that immune cells and brain cells can be similarly influenced (Provençal et al., 2012; Massart et al., 2016a). Increased methylation of DNA in promoter regions of a gene has been reported to suppress transcription and gene expression (Levine et al., 1991; Tate and Bird, 1993). Alternatively, increased methylation of DNA in gene body regions (i.e., introns and exons) may increase gene expression (Hellman and Chess, 2007). Therefore, characterization of methylation status of DNA in promoter and gene body regions may provide insight regarding the influence of prenatal environment on postnatal phenotype. Beef cattle health, animal welfare/well-being, behavior, and production trait phenotypes are important to producers and consumers. This study investigated how environmental stressors incurred by gestating dams could affect relevant traits in beef cattle, by evaluating the influence of prenatal transportation stress on genome-wide DNA methylation in their offspring. We hypothesized that prenatal transportation stress would alter genome-wide methylation of DNA from white blood cells (WBC) of 28-d-old Brahman bull calves.

MATERIALS AND METHODS

All experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010)

and were approved by the Texas A&M University Animal Care and Use Committee.

Animal Procedures

Multiparous Brahman cows were assigned to 1 of 2 treatment groups (**Transported**: $n = 48$ and **Control**: $n = 48$) according to age, parity, and temperament (Littlejohn et al., 2016). Transported cows were hauled for a 2-h duration at 60 ± 5 , 80 ± 5 , 100 ± 5 , 120 ± 5 , and 140 ± 5 d of gestation (Price et al., 2015). Control cows were maintained in the same manner as Transported cows with the exception of being transported. The 2 groups were housed in the same pasture (at the Texas A&M AgriLife Research and Extension Center at Overton, TX) and fed the same diet (Littlejohn et al., 2016). From these cows, 26 male and 18 female calves (Control group) were born to Control dams, while 20 male and 21 female calves [prenatally stressed (**PNS**) group] were born to Transported dams. Male calves were maintained as bulls throughout the study. Each calf was restrained manually at 28 d of age for less than 5 min for blood sample collection. One 10-mL vacuum tube (BD, Franklin Lakes, NJ) containing EDTA was used for blood sample collection from each calf via jugular venipuncture with a sterile 18-gauge needle. The WBC were isolated and stored at -80°C until DNA was extracted. For methylation analysis, samples from bull calves were stratified into groups based on the following criteria: 1) adequate availability of DNA, 2) calf treatment group, and 3) calf sire. From those stratified groups, samples from 7 PNS and 7 Control bull calves were randomly selected for this study. Our lab chose to focus on bull calves rather than heifer calves because of the reported transgenerational impact of PNS attributed to the male germline (Rodgers et al., 2013; Rodgers and Bale, 2015).

Sample Analysis

Processing of blood samples. Blood samples were centrifuged at $2,671 \times g$ for 30 min at 6°C . The WBC layer was isolated and transferred into 2-mL nuclease-free microcentrifuge tubes. Samples were repeatedly washed in red blood cell lysis buffer solution until a clean cell pellet was produced. Specifically, one wash included the following: red blood cell lysis buffer was added to the tube, mixed by vortex for 20 s, shaken for 3 min, centrifuged at $1600 \times g$ for 5 min, and the supernatant was discarded. Clean WBC pellets were stored at -80°C until they were thawed for DNA extraction.

DNA extraction. Phenol-chloroform extraction procedures were used to isolate DNA from each WBC pellet for methylation analysis. The following description summarizes extraction procedures: Samples were removed from the -80°C freezer and placed on wet ice for thawing and kept on ice between procedural steps. White blood cell pellets were homogenized in extraction buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5), to which 10 mg/mL proteinase K and 20% SDS was added for proteinase K digestion and incubated at 55°C for 2 h. Samples were extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and twice with an equal volume of 1-bromo-3-chloropropane (substituted for chloroform). After each extraction, samples underwent centrifugation for 5 min at $8,000 \times g$. DNA was precipitated by addition of 10% 3 M sodium acetate (pH 5.2) and 1 volume isopropanol to the solution, followed by centrifugation for 5 min at $13,000 \times g$. Pelleted DNA was rinsed with 70% ethanol, centrifuged at $13,000 \times g$ for 5 min, air-dried, rinsed with 95–100% ethanol, centrifuged, air-dried, and suspended in 150–200 μL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Purified DNA was stored at -80°C until selected samples were shipped to Zymo Research Corp (Irvine, CA) for DNA methylation analysis.

Library construction for DNA methylation analysis. A method of reduced representation bisulfite sequencing (RRBS), Methyl-MiniSeq (Zymo Research; Irvine, CA), was used to assess differential DNA methylation in samples from PNS compared with Control bull calves. Libraries were prepared from 200–500 ng of genomic DNA digested with 60 units of Taq α I and 30 units of MspI (NEB) sequentially and then extracted with Zymo Research (ZR) DNA Clean & Concentrator-5 kit (Cat#: D4003). Fragments were ligated to preannealed adapters containing 5'-methyl-cytosine instead of cytosine according to Illumina's specified guidelines (www.illumina.com). Adaptor-ligated fragments of 150–250 bp and 250–350 bp in size were recovered from a 2.5% NuSieve 1:1 agarose gel (Zymoclean Gel DNA Recovery Kit, ZR Cat#: D4001). The fragments were then bisulfite-treated using the EZ DNA Methylation-Lightning Kit (ZR, Cat#: D5020). Preparative-scale PCR was performed and the resulting products were purified (DNA Clean & Concentrator - ZR, Cat#D4005) and sequenced on an Illumina HiSeq.

Methyl-MiniSeq sequence alignments and data analysis. Sequence reads from bisulfite-treated EpiQuest libraries were identified with standard Illumina base-calling software and analyzed via a

Zymo Research proprietary analysis pipeline, which is written in Python and used Bismark (<http://www.bioinformatics.babraham.ac.uk/projects/bismark/>) to perform the alignment. Bismark is used to map bisulfite converted sequence reads to determine methylation status of cytosine nucleotides. Index files were constructed using the bismark_genome_preparation command (creates a subdirectory in the fasta file directory) and the entire reference genome. The --non_directional parameter was applied while running Bismark. All other parameters were set to default. Filled-in nucleotides were trimmed off for methylation calling. Methylation ratio was defined as the measured number of cytosines (number of reads reporting a C) divided by the total number of cytosines (total number of reads reporting a C or T) covered at that site. Each methylation difference was calculated by subtracting the average Control methylation ratio at a site from the average PNS methylation ratio at a site. Fisher's exact test or *t*-test was performed for each CpG site which has at least 5 reads coverage, and promoter, gene body and CpG island annotations were added for each CpG included in the comparison.

Prediction of pathways and functions altered by prenatal stress. An enrichment analysis was executed (analyzed on April 3, 2018) using Ingenuity Pathway Analysis software (IPA; Redwood City, CA) to assess alterations to signaling pathways and biological functions in PNS compared to Control bull calves by overlaying genes with differentially methylated (i.e., at least 10% more or less methylated than Controls) CpG sites onto networks generated by IPA. All *P* values (*P* values of overlap) for those pathways and functions were calculated using a right-tailed Fisher exact test. These measured the probability of association of the significant genes with a pathway or function group by random chance alone. Only differentially methylated CpG sites ($P \leq 0.05$) that had a methylation difference of $\geq 10\%$ and were located within promoter regions were utilized in the enrichment analysis. The promoter region was selected, because DNA methylation in the promoter region regulates gene activity. Specifically, increased DNA methylation within gene promoter regions has been reported to cause suppressed transcription and gene expression (Levine et al., 1991; Tate and Bird, 1993). Therefore, for the purpose of predicting alterations to signaling pathways, hypomethylation within gene promoters was assumed to result in increased gene activity, while hypermethylation within gene promoters was assumed to result in decreased gene

activity. It is important to note that genes can be inhibitors or activators of specific cellular processes. Therefore, regulation of gene expression by increased or decreased DNA methylation can result in activation or inhibition of cellular processes, depending on the function of that specific gene. For example, gene expression that is down-regulated by increased DNA methylation in an inhibitory gene could result in activation of a cellular process.

RESULTS AND DISCUSSION

Genome-wide DNA Methylation in PNS and Control Bull Calves

Coverage summary. Samples from Control and PNS calves had an average total read number of 38,534,348 and 34,894,063 read pairs, respectively. Both Control and PNS samples had an average mapping efficiency of 33% and bisulfite conversion rate of 99%. Sequence depths of unique CpG sites were 6,392,121 (9.0 times) for samples from Control and 6,358,081 (8.2 times) for samples from PNS bull calves on average. Sequence depths of unique CHG sites were 11,046,912 (7.3 times) for samples from Control and 10,985,061 (6.6 times) for samples from PNS bull calves on average. Sequence depths of unique CHH sites were 27,388,851 (6.9 times) for samples from Control and 27,218,778 (6.3 times) for samples from PNS bull calves on average.

CpG sites. A summary of genome-wide distribution of differential DNA methylation in CpG sites (i.e., in the CpG context; defined as a cytosine followed by a guanine) across promoters, introns, exons, and intergenic regions in PNS compared with Control bull calves is located in [Table 1](#). Briefly, 16,128 CpG sites (52.76% of which were located within CpG islands) were differentially methylated in PNS compared to Control bull calves ($P \leq 0.05$). The majority of differentially methylated CpG sites were found in intergenic regions (65.5%), followed by introns (19.2%), exons (7.8%), and promoters (7.5%). Of those affected sites, 45.93% were hypermethylated (at least 10% more methylated than Controls; $P \leq 0.05$) and 54.07% were hypomethylated (at least 10% less methylated than Controls; $P \leq 0.05$). Methylation of DNA in mammals primarily occurs within CpG contexts ([Ehrlich et al., 1982](#)), although reports of substantial DNA methylation within non-CpG contexts (i.e., in the CHG or CHH context; in which C = cytosine; G = guanine; H = either adenine, thymine, or cytosine) have been established in embryonic stem cells, gametes, and brain cells ([Ramsahoye et al., 2000](#); [Shirane](#)

[et al., 2013](#); [Varley et al., 2013](#)). Previously, methylation of cytosine nucleotides in non-CPG contexts was thought to vanish upon cellular differentiation ([Lister et al., 2009](#)). However, methylation of DNA in non-CpG contexts of differentiated mammalian cell types has gained attention in various tissues in recent years ([Ziller et al., 2011](#); [Barua et al., 2014](#); [Zhou et al., 2016](#)).

Non-CpG sites. A summary of genome-wide distribution of differential DNA methylation in CHG sites across promoters, introns, exons, and intergenic regions in PNS compared with Control bull calves is located in [Table 1](#). Briefly, 226 CHG sites (20.28% of which were located within CpG islands) were differentially methylated in PNS compared to Control bull calves ($P \leq 0.05$). The majority of differentially methylated CHG sites were found in intergenic regions (64.6%), followed by introns (24.3%), exons (6.6%), and promoters (4.4%). Of those affected sites, 47.79% were hypermethylated and 52.21% were hypomethylated.

A summary of genome-wide distribution of differential DNA methylation in CHH sites across promoters, introns, exons, and intergenic regions in PNS compared with Control bull calves is located in [Table 1](#). Briefly, 391 CHH sites (15.20% of which were located within CpG islands) were differentially methylated in PNS compared to Control bull calves ($P \leq 0.05$). The majority of differentially methylated CHH sites were found in intergenic regions (62.4%), followed by introns (30.9%), promoters (3.6%), and exons (3.1%). Of those affected sites, 53.45% were hypermethylated and 46.55% were hypomethylated.

[Zhou et al. \(2016\)](#) assessed DNA methylation of 10 bovine cell types using RRBS and found that of the CpG-enriched regions, 33.5% were in the CpG context, 1.1% were in the CHG context, and 1.5% were in the CHH context. Of the cytosines that were differentially methylated compared to one or more of the other 10 cell types, 94.34% were in the CpG context and 5.66% were in the non-CpG context ([Zhou et al., 2016](#)). Furthermore, [Barua et al. \(2014\)](#) reported differential methylation of CHG and CHH contexts due to prenatal programming in rodents. Thus, increasing evidence suggests an influence of DNA methylation in non-CpG contexts in differentiated mammalian cells.

Differential DNA Methylation in CpG Sites

Promoter regions. A total of 1,205 differentially methylated CpG sites were identified within promoter regions in PNS compared with Control bull

Table 1. Summary of genome-wide distribution of differential DNA methylation (HYPER=Hypermethylation, HYPO=Hypomethylation) across CpG, CHG, and CHH sites in prenatally stressed (PNS) compared with Control bull calves^{1,2,3}

Genomic region	Affected regions (N)	Percent of total regions affected	HYPER regions (N)	⁴ Percent of affected regions with HYPER	HYPO regions (N)	Percent of affected regions with HYPO	HYPER regions within CpG Islands (N)	Percent of HYPER regions within CpG Islands	HYPO regions within CpG Islands (N)	Percent of HYPO regions within CpG Islands
CpG Sites										
Promoter	1,205	7.5%	543	3.37%	662	4.10%	307	4.14%	364	4.17%
Intron	3,103	19.2%	1,386	8.59%	1,717	10.65%	303	4.09%	354	4.06%
Exon	1,260	7.8%	602	3.73%	658	4.08%	364	4.91%	383	4.39%
Intergenic	10,560	65.5%	4,876	30.23%	5,684	35.24%	1,025	13.84%	1,146	13.14%
Total	16,128	100.0%	7,407	45.93%	8,721	54.07%	1,999	26.99%	2,247	25.77%
CHG Sites										
Promoter	10	4.4%	3	1.33%	7	3.10%	1	0.93%	2	1.69%
Intron	55	24.3%	28	12.39%	27	11.95%	1	0.93%	2	1.69%
Exon	15	6.6%	9	3.98%	6	2.65%	3	2.78%	2	1.69%
Intergenic	146	64.6%	68	30.09%	78	34.51%	5	4.63%	7	5.93%
Total	226	100.0%	108	47.79%	118	52.21%	10	9.26%	13	11.02%
CHH Sites										
Promoter	14	3.6%	6	1.53%	8	2.05%	2	0.96%	1	0.55%
Intron	121	30.9%	65	16.62%	56	14.32%	4	1.91%	5	2.75%
Exon	12	3.1%	6	1.53%	6	1.53%	2	0.96%	1	0.55%
Intergenic	244	62.4%	132	33.76%	112	28.64%	10	4.78%	5	2.75%
Total	391	100.00%	209	53.45%	182	46.55%	18	8.61%	12	6.59%

¹Affected regions were considered $P \leq 0.05$.

²In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

³Differentially methylated sites were at least 10% more or less methylated than Controls.

⁴Percent of hypermethylated regions and percent of hypomethylated regions add up to 100%.

calves (Table 1). A greater percentage of these CpG sites were hypomethylated (54.07%) compared to hypermethylated (45.93%; Table 1). Richetto et al. (2017) reported a similar occurrence in mice that were exposed to a prenatal viral challenge on gestational day 9 or 17, with 64% and 61% of differentially methylated regions being hypomethylated and 36% and 39% being hypermethylated, respectively. Differential methylation within promoter regions in PNS compared with Control bulls was distributed throughout the genome, with all chromosomes having both hypomethylated and hypermethylated CpG sites (Fig. 1). The number of hypermethylated compared with hypomethylated CpG sites within promoter regions in PNS compared with Control bulls is represented in Fig. 1. Increased DNA methylation within gene promoter regions has been reported to cause suppressed transcription and gene expression (Levine et al., 1991; Tate and Bird, 1993). This suggests that hypermethylated CpG sites within gene promoter regions might have downregulated gene expression and hypomethylated CpG sites within gene promoter regions might have upregulated

gene expression in PNS calves compared with Controls.

Strongly differentially methylated CpG sites (“strongly” being defined as a degree of methylation in PNS calves that was at least 33% different from Controls) were specifically examined to highlight those sites that exhibited the greatest changes in degree of methylation due to PNS treatment. In agreement with this study’s overall findings, a greater percentage of strongly hypomethylated (methylation difference ≤ -0.33 ratio) compared to strongly hypermethylated (methylation difference ≥ 0.33 ratio) CpG sites were identified within promoter regions ($P \leq 0.05$) in PNS bull calves compared with Controls. Six strongly hypermethylated CpG sites were located within promoter regions in PNS compared with Control calves (Table 2). Among these were 2 CpG sites that were located within the promoter region of the *NQO2* gene. This hypermethylated gene was also involved in the upregulation of the “NRF2-mediated Oxidative Stress Response” pathway in PNS calves (Supplementary Table S1). Furthermore, a base pair deletion in the promoter region of this gene

Figure 1. Comparison of hypermethylated and hypomethylated CpG sites within promoter regions in prenatally stressed (PNS) compared with Control bull calves.

Table 2. Strongly hypermethylated (methylation difference ≥ 0.33 ratio) CpG sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	P value
chr7 [†]	<i>CCDC105</i>	9178140	9178141	–	7.86	8.71	0.36	0.003097
chr11 ^{†*}	<i>MRPL41</i>	105596122	105596123	–	8.57	7.00	0.35	0.003315
chr12 [†]	<i>PCDH17</i>	5200175	5200176	–	11.14	7.29	0.34	0.0004446
chr21 [*]	<i>MIR655</i>	67587446	67587447	+	8.57	9.43	0.45	0.008982
chr23	<i>NQO2</i>	50497771	50497772	–	6.71	5.86	0.39	0.002682
chr23 [†]	<i>NQO2</i>	50497904	50497905	–	6.71	5.86	0.42	0.02622

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CPG island.

^{*}DNA methylation was exclusively located within the promoter region.

has been associated with schizophrenia in humans (Harada et al., 2003), suggesting the potential for physiological and behavioral alterations due to differences in neurotransmitter pathways.

The top (lowest P values) 30 of 543 significantly ($P \leq 0.05$) hypermethylated CpG sites located within promoter regions in PNS compared with Control calves are listed in Table 3. Among these was a CpG site within the promoter region of the Caudal Type Homeobox 2 (*CDX2*) gene. *CDX2* is involved in early embryo pluripotency, differentiation, and development (Xie et al., 2013). Therefore, it is consistent that this gene was also involved in the alterations to the pathways, “Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency, Transcriptional Regulatory Network in Embryonic Stem Cells, Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency” in PNS calves (Supplementary Table S1). In vitro hyperosmolar stress resulted in reduced *CDX2* and inhibited potency in the early embryo of the mouse (Xie et al., 2013). Bovine embryos exposed to heat

stress exhibited reduced *CDX2* gene expression in total blastocyst RNA (Silva et al., 2013). These studies agree with the current study, which is predictive of downregulated expression of *CDX2* due to increased DNA methylation in the promoter region of *CDX2* in PNS bulls compared with Controls.

There were 22 strongly hypomethylated CpG sites located within promoter regions in PNS compared with Control calves (Table 4). Among these was a CpG site within the promoter region of the Guanine Nucleotide-Binding Protein G(S) Subunit Alpha (*GNAS*) gene. In cattle and other mammals, *GNAS* is an imprinted gene that has been reported to play a major role in development, growth, and metabolism (Plagge et al., 2004; Sikora et al., 2011). DNA methylation of a paternally expressed transcript of this gene, *GNASXL*, has been reported to be positively associated with prenatal maternal stress (as quantified by increased depression, anxiety, and cortisol) in humans (Vangeel et al., 2015). This is in contrast with the hypomethylated CpG site within the promoter region of the *GNAS* gene in PNS bull calves in this study.

Table 3. Top (lowest *P* values) 30 hypermethylated CpG sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr1 [†]	<i>CLSTN2</i>	130108179	130108180	–	11.71	13.00	0.11	0.00009504
chr1	<i>CLDN8</i>	5036161	5036162	+	16.57	14.29	0.13	0.0003032
chr2 [†]	<i>TMEM200B</i>	125008770	125008771	+	12.43	9.71	0.13	0.00028
chr3 [†]	<i>SHE</i>	16143944	16143945	+	7.14	12.29	0.18	0.0009009
chr7 [†]	<i>HNRNPM</i>	18289571	18289572	–	9.43	8.71	0.21	0.0002804
chr7 [†]	<i>HNRNPM</i>	18289557	18289558	–	9.43	8.71	0.22	0.0006671
chr7	<i>SIL1</i>	52143957	52143958	+	13.00	10.71	0.1	0.001113
chr7 [†]	<i>HNRNPM</i>	18289508	18289509	–	9.43	8.57	0.21	0.001569
chr7 [†]	<i>HNRNPM</i>	18289493	18289494	–	9.29	8.71	0.22	0.001877
chr7 [†]	<i>HNRNPM</i>	18289497	18289498	–	9.43	8.71	0.22	0.001877
chr7 [†]	<i>HNRNPM</i>	18289505	18289506	–	9.43	8.71	0.22	0.001877
chr10*	<i>EIF3J</i>	103983752	103983753	+	9.43	8.86	0.13	6.067E–07
chr10 ^{†*}	<i>SIX6</i>	72952940	72952941	–	13.00	10.14	0.19	0.0001318
chr10 [†]	<i>SHC4</i>	61503850	61503851	+	8.86	9.14	0.17	0.001072
chr10*	<i>EIF3J</i>	103983746	103983747	+	9.29	8.86	0.14	0.001454
chr10	<i>SHC4</i>	61504208	61504209	–	9.86	7.29	0.15	0.001506
chr10 [†]	<i>LHFPL2</i>	9527046	9527047	+	13.00	8.86	0.11	0.001618
chr11	<i>BRE, RBKS</i>	71827429	71827430	–	12.14	10.86	0.3	0.0007929
chr12*	<i>CDX2</i>	32316737	32316738	–	18.86	13.00	0.25	0.0002012
chr12 [†]	<i>PCDH17</i>	5200175	5200176	–	11.14	7.29	0.34	0.0004446
chr13 [†]	<i>BMP7</i>	59425545	59425546	–	8.00	8.00	0.11	0.001394
chr15	<i>MMP7</i>	6390211	6390212	+	8.57	6.29	0.19	0.000205
chr15 [†]	<i>CHRM4</i>	77253713	77253714	+	10.71	6.14	0.13	0.000309
chr16 ^{†*}	<i>MMP23B</i>	52263865	52263866	+	10.71	8.86	0.12	0.001214
chr16 ^{†*}	<i>SLC45A1</i>	45908696	45908697	–	11.57	9.00	0.11	0.001281
chr19	<i>ITGA3</i>	37230331	37230332	+	12.29	11.00	0.12	0.0000896
chr19*	<i>CORO6</i>	21435132	21435133	+	21.00	18.00	0.17	0.001546
chr25 [†]	<i>METRNL</i>	613852	613853	–	8.71	6.00	0.12	0.001253
chr26	<i>ZWINT</i>	2851210	2851211	+	10.00	11.71	0.15	0.0001167
chr28 [†]	<i>TRIM67</i>	3519622	3519623	+	18.14	19.14	0.11	0.001037

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CPG island.

*DNA methylation was exclusively located within the promoter region.

The top (lowest *P* values) 30 of 662 significantly ($P \leq 0.05$) hypomethylated CpG sites located within promoter regions in PNS compared with Control calves are listed in Table 5. Among these was a CpG site within the promoter region of the Dopamine Receptor D1 (*DRD1*) gene. This gene has been associated with behavioral disorders such as psychosis and schizophrenia (Andreou et al., 2016). The *DRD1* gene was involved in the following pathways altered in PNS calves in this study: “cAMP-mediated signaling, G-Protein Coupled Receptor Signaling, Gαs Signaling, Dopamine-DARPP32 Feedback in cAMP Signaling, CDK5 Signaling, Gap Junction Signaling, and Dopamine Receptor Signaling” (Supplementary Table S1). Rat pups that were separated from their mothers for 6-h periods each day during the first 2 wk of life had downregulated *DRD1* gene expression in the

nucleus accumbens (Zhu et al., 2010). Rat pups that were separated from their mothers for 3-h periods had decreased mid-brain tyrosine hydroxylase-immunoreactive dopaminergic neurons as juveniles (15 d of age) but increased numbers as adolescents (35 d of age) and adults (70 d of age; Chocyk et al., 2011). These varying results suggest that alterations to the *DRD1* gene due to prenatal or early life stress are specific to life stage.

Gene body regions. Differential methylation of 4,363 CpG sites were identified within gene body (i.e., introns and exons) regions in PNS compared with Control bull calves (Table 1). A greater percentage of these CpG sites were hypomethylated (Table 1). Differential methylation within gene body regions in PNS compared with Control bulls was distributed throughout the genome, with both

Table 4. Strongly hypomethylated (methylation difference ≤ -0.33 ratio) CpG sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	P value
chr1*	<i>EPCAM</i>	157207666	157207667	–	7.4	8.9	–0.34	0.0495
chr1**	<i>GNAS</i>	154101009	154101010	–	10.6	5.4	–0.35	0.0147
chr7*	<i>TNFRSF10D</i>	18053077	18053078	–	10.9	16.0	–0.38	0.01531
chr8	<i>KHNYN, CBLN3</i>	104517901	104517902	–	11.9	9.0	–0.35	0.03365
chr9	<i>PLA2G4B</i>	103674611	103674612	+	14.7	6.9	–0.44	0.04478
chr11	<i>ATF6B</i>	103182975	103182976	+	13.7	9.4	–0.33	0.002325
chr11†	<i>SNX19</i>	100835712	100835713	–	8.0	11.4	–0.35	0.006669
chr13‡	<i>LOXL4</i>	58167618	58167619	–	12.7	12.4	–0.34	0.01073
chr13‡	<i>MGRN1</i>	58167617	58167618	+	11.3	23.0	–0.33	0.004263
chr13‡	<i>YDJC</i>	54817882	54817883	–	6.7	10.9	–0.39	0.007604
chr17‡	<i>YDJC</i>	73172496	73172497	–	7.3	12.0	–0.45	0.009104
chr19	<i>ATF6B</i>	22423681	22423682	–	14.3	14.0	–0.38	0.01513
chr20*	<i>CSNK1D</i>	69935936	69935937	–	9.0	9.4	–0.39	0.01086
chr21**	<i>GNAS</i>	30886861	30886862	–	11.3	9.6	–0.33	0.01602
chr23‡	<i>MGRN1</i>	51619275	51619276	–	11.0	8.6	–0.33	0.004263
chr23‡	<i>SNX19</i>	17940352	17940353	–	9.4	11.1	–0.35	0.02976
chr23‡	<i>TMOD1</i>	51239299	51239300	+	11.6	9.0	–0.35	0.0389
chr23*	<i>UBXN8</i>	48211861	48211862	–	10.9	6.3	–0.37	0.001421
chr24‡	<i>MGRN1</i>	3104521	3104522	–	10.9	7.0	–0.33	0.004263
chr25*	<i>CARD9</i>	1721752	1721753	–	9.7	9.7	–0.34	0.002587
chr25	<i>MIR99B, MIRLET7E</i>	4471948	4471949	+	8.4	6.3	–0.38	0.005474
chr25*	<i>TNFRSF10D</i>	2238087	2238088	+	12.3	9.1	–0.37	0.02807

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

†DNA methylation was located within a CPG island.

*DNA methylation was exclusively located within the promoter region.

hypomethylation and hypermethylation observed in CpG sites across all chromosomes (Fig. 2). The number of hypermethylated compared with hypomethylated CpG sites within gene body regions in PNS compared with Control bulls are represented in Fig. 2. Increased DNA methylation in gene bodies may result in activated gene expression (Hellman and Chess, 2007), suggesting that hypermethylated gene body regions might have upregulated gene expression and hypomethylated gene body regions might have downregulated gene expression.

The top (lowest *P* values) 30 of 1,988 significantly ($P \leq 0.05$) hypermethylated CpG sites located within gene body regions in PNS compared with Control calves are listed in Table 6. Among these was a CpG site within the gene body region of the Iodothyronine Deiodinase 3 (*DIO3*) gene, suggesting a potential upregulation in gene expression. The *DIO3* gene is an imprinted gene in mice, cattle, and other species that encodes a protein, which inactivates thyroid hormones (Tsai et al., 2002; Yang et al., 2017). Thyroid hormones influence central nervous system development

(Bernal, 2005) and behavior (Stohn et al., 2018). Male and female mice deficient in *DIO3* exhibited increased aggression in response to an intruder (Stohn et al., 2018). Furthermore, rats whose dams consumed ethanol from day 8 to 21 of gestation had increased placental *DIO3* mRNA compared to Controls (Shukla et al., 2011). These studies suggest that prenatal environment can shape nervous system development through alterations to the *DIO3* gene.

The top (lowest *P* values) 30 of 2,375 significantly ($P \leq 0.05$) hypomethylated CpG sites located within gene body regions in PNS compared with Control calves are listed in Table 7. Among these was a CpG site within the gene body region of the Adenylate Cyclase Activating Polypeptide 1 (*ADCYAP1*) gene, suggesting a potential downregulation in gene expression. The *ADCYAP1* gene encodes for the PACAP peptide and is associated with stress disorders and posttraumatic stress disorder (Ressler et al., 2011). Mice that were deficient in PACAP exhibited behavioral abnormalities such as hyperactivity, jumping, and depression

Table 5. Top (lowest *P* values) 30 hypomethylated CpG sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr3 [†]	<i>ECHDC2</i>	94016695	94016696	+	9.14	12.29	-0.28	0.0006247
chr4 [†]	<i>OSBPL3</i>	71296952	71296953	+	17.29	15.57	-0.13	0.001308
chr7	<i>FDX1L</i>	16072432	16072433	+	9.43	9.57	-0.18	0.0004641
chr7 [†]	<i>NMRK2</i>	21294100	21294101	+	10.00	7.43	-0.16	0.000824
chr8	<i>MIR2887-2, MIR2887-1, MIR2904-1, MIR2904-3, MIR2904-2</i>	59158057	59158058	+	9.71	13.57	-0.23	0.0008405
chr8 ^{†*}	<i>XKR6</i>	8255834	8255835	-	14.57	7.43	-0.11	0.001542
chr9 [*]	<i>SEC63</i>	42650623	42650624	+	14.14	9.29	-0.17	0.0002254
chr9	<i>OPRM1</i>	92152196	92152197	+	8.14	9.43	-0.13	0.0002767
chr9	<i>OPRM1</i>	92152194	92152195	+	8.14	9.43	-0.2	0.001245
chr10 [*]	<i>DRD1</i>	5653417	5653418	+	9.14	7.86	-0.19	0.0003017
chr10	<i>LTB4R</i>	20689223	20689224	-	8.43	9.86	-0.16	0.0007834
chr11 [†]	<i>SIX2</i>	27263419	27263420	+	21.00	17.00	-0.11	0.0005395
chr11 ^{†*}	<i>SURF4</i>	104335032	104335033	-	11.29	11.14	-0.14	0.001013
chr15 [†]	<i>APLN</i>	81738036	81738037	+	25.57	24.57	-0.23	0.0004384
chr18 [*]	<i>EGLN2</i>	50330819	50330820	-	8.00	6.57	-0.15	0.0007258
chr18 ^{†*}	<i>NAT14</i>	62474955	62474956	-	11.57	10.86	-0.25	0.001256
chr18	<i>FUZ</i>	56625931	56625932	-	10.57	8.43	-0.3	0.001281
chr19 [†]	<i>PMP22</i>	33382476	33382477	-	11.43	12.57	-0.12	0.00007101
chr19	<i>ERBB2</i>	40722503	40722504	+	13.14	9.57	-0.1	0.0004212
chr19 [*]	<i>MPDU1</i>	27924874	27924875	-	8.14	6.57	-0.15	0.001674
chr21 ^{†*}	<i>NKX2-8</i>	47199004	47199005	+	12.86	14.86	-0.24	0.0003029
chr21 [*]	<i>CHRNA7</i>	30181352	30181353	-	11.00	7.29	-0.29	0.0005365
chr21 [†]	<i>CSPG4</i>	33574762	33574763	-	7.14	6.86	-0.17	0.00072
chr22 [†]	<i>ITGA9</i>	10946832	10946833	-	17.00	17.43	-0.26	0.001275
chr25 [†]	<i>PGP</i>	1746496	1746497	+	18.57	17.57	-0.25	0.001152
chr26	<i>MIR2397</i>	49743379	49743380	+	12.43	9.57	-0.14	0.00004049
chr26	<i>PRLHR</i>	39220962	39220963	+	11.43	9.29	-0.22	0.00008747
chr27 [*]	<i>UBXN8</i>	26008284	26008285	-	10.00	8.57	-0.37	0.001421
chr29 [*]	<i>TALDO1</i>	50864154	50864155	-	8.57	8.14	-0.27	0.0001148
chr29 [*]	<i>AP2A2</i>	50445180	50445181	+	10.71	7.71	-0.2	0.0004282

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CPG island.

^{*}DNA methylation was exclusively located within the promoter region.

(Ishihama et al., 2010). Furthermore, mice whose dams were exposed to a posttraumatic stress disorder model (restrained for 2-h period and then forced to swim for a 20-min period with 6 other rats) had downregulated *ADCYAP1* gene expression (Zhang et al., 2016). These studies support the potential influence of prenatal stress on the *ADCYAP1* gene in this study and suggest altered behavioral effects due to prenatal stress. Behavioral alterations were previously observed in PNS calves (Littlejohn et al., 2016).

Differential DNA Methylation in CHG Sites

Promoter regions. There were 10 differentially methylated CHG sites within promoter regions in PNS compared with Control bull calves (Table 1).

A greater percentage of these CHG sites were hypomethylated compared to hypermethylated (Table 1).

The 3 significantly ($P \leq 0.05$) hypermethylated CHG sites located within promoter regions in PNS compared with Control calves are listed in Table 8. Among these was a CHG site within the promoter region of the Crystallin Beta B3 (*CRYBB3*) gene. The *CRYBB3* gene is a member of the crystallin gene family, dysregulations of which have been associated with neural disorders, such as Alzheimer's disease and schizophrenia (Shinohara et al., 1993; Middleton et al., 2002). Crystallin family heat shock proteins likely have a neuroprotective function (Masilamoni et al., 2006; Ousman et al., 2007). Specifically, *CRYBB3* was upregulated in 3 models of mice whose dams underwent immune activation during gestation (dams were administered human

Figure 2. Comparison of hypermethylated and hypomethylated CpG sites within gene body regions in prenatally stressed (PNS) compared with Control bull calves.

influenza virus, poly (I:C), or recombinant IL-6 during gestation; Garbett et al., 2012). Previous studies suggest differential methylation of the *CRYBB3* gene might result in alterations in neural and behavioral characteristics of PNS calves.

The 7 significant ($P \leq 0.05$) hypomethylated CHG sites located within promoter regions in PNS compared with Control calves are listed in Table 9. Among these was a CHG site within the promoter region of the Coagulation Factor II Receptor-Like 3 (*F2RL3*) gene. The *F2RL3* gene is hypomethylated in adult smokers and has been associated with mortality risk due to cardiovascular diseases (Breitling et al., 2012).

Gene body regions. There were 70 differentially methylated CHG sites within gene body regions in PNS compared with Control bull calves (Table 1). A slightly greater percentage of these CHG sites were hypermethylated compared to hypomethylated (Table 1).

The top (lowest P values) 30 of 37 significantly ($P \leq 0.05$) hypermethylated CHG sites located within gene body regions in PNS compared with Control calves are listed in Table 10. Among these was a CHG site within the gene body region of the Protein Kinase C Alpha (*PRKCA*) gene. The *PRKCA* gene encodes for Protein Kinase C Alpha, a member of the serine- and threonine-specific protein kinases, which phosphorylates peptide substrates and is involved in various cell-signaling processes (Lee et al., 2017). The *PRKCA* gene has been associated with PTSD and memory in humans (De Quervain et al., 2012). Furthermore, rats whose dams were stressed by placing them on an elevated platform made of Plexiglass twice each

day for a 10-min period between 12 and 16 d of gestation resulted in genome-wide alterations in gene expression, including expression of *PRKCA* (Mychasiuk et al., 2011). Due to its influence on various cell-signaling pathways, differential methylation of *PRKCA* has the potential to influence many biological processes in PNS calves.

The top (lowest P values) 30 of 33 significantly ($P \leq 0.05$) hypomethylated CHG sites located within gene body regions in PNS compared with Control calves are listed in Table 11. Among these was a CHG site within the gene body region of the Diacylglycerol Acyltransferase 1 (*DGATI*) gene. The *DGATI* gene encodes for a protein enzyme involved in the conversion of diacylglycerol and fatty acyl CoA to triacylglycerol diacylglycerol and has been associated with metabolic diseases (Yen et al., 2008) and with milk production traits in dairy cattle (Mohammed et al., 2015). Mice whose dams underwent daily restraint stress for a 3-h period from 8 d of gestation to birth had increased accumulation of lipids in the liver and increased *DGATI* gene expression (Maeyama et al., 2015). Differential methylation of *DGATI* suggests an influence of pre-natal stress on postnatal metabolic processes.

Differential DNA Methylation in CHH Sites

Promoter regions. There were 14 differentially methylated CHH sites within promoter regions in PNS compared with Control bull calves (Table 1). A greater percentage of these CHH sites were hypomethylated compared to hypermethylated (Table 1).

The 6 significantly ($P \leq 0.05$) hypermethylated CHH sites located within promoter regions in PNS compared with Control calves are listed in Table 12.

Table 6. Top (lowest *P* values) 30 hypermethylated CpG sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr1 [§]	<i>CLDN8</i>	5036161	5036162	+	16.57	14.29	0.13	0.0003032
chr1 ^{†‡}	<i>CLSTN2</i>	130108179	130108180	–	11.71	13.00	0.11	0.00009504
chr1 ^{†‡}	<i>EPHB1</i>	135353859	135353860	–	12.43	12.29	0.16	0.00003959
chr2 ^{†‡}	<i>TMEM200B</i>	125008770	125008771	+	12.43	9.71	0.13	0.00028
chr3 ^{†§}	<i>DMRTA2</i>	96456243	96456244	–	19.29	15.29	0.15	0.0003634
chr3 ^{†‡}	<i>C3H1orf52</i>	59027290	59027291	–	6.29	8.71	0.15	0.00001461
chr4 ^{†‡}	<i>NUDCD3</i>	77653827	77653828	–	5.86	6.29	0.4	0.0004187
chr5 ^{†‡}	<i>ZNF384</i>	104130929	104130930	+	7.71	8.14	0.15	0.0003626
chr7 [§]	<i>F12</i>	40258637	40258638	–	7.71	5.71	0.13	0.0004367
chr7 [§]	<i>HNRNPM</i>	18289571	18289572	–	9.43	8.71	0.21	0.0002804
chr7 ^{†‡}	<i>CRTC1</i>	4435796	4435797	+	11.29	10.14	0.14	0.0004044
chr11 [§]	<i>SLC8A1</i>	22824023	22824024	+	9.57	8.86	0.29	0.0001272
chr11 ^{†‡}	<i>EHMT1</i>	105461042	105461043	+	7.29	6.57	0.63	0.0003017
chr11 ^{†‡}	<i>RALGDS</i>	103166859	103166860	–	7.14	6.29	0.55	0.00003935
chr11 ^{†‡}	<i>RALGDS</i>	103166862	103166863	–	7.14	6.29	0.48	0.0001696
chr11 ^{†‡}	<i>RALGDS</i>	103166855	103166856	–	7.14	6.29	0.55	0.0003
chr12 ^{†‡‡}	<i>CARS2</i>	89257092	89257093	–	11.71	9.00	0.2	0.0001168
chr12 ^{†‡}	<i>DOCK9</i>	79833906	79833907	+	10.00	6.00	0.2	0.0002803
chr12 ^{†‡‡}	<i>GRTPI</i>	90628533	90628534	–	6.57	6.43	0.11	0.0004244
chr15 [§]	<i>CHRM4</i>	77253713	77253714	+	10.71	6.14	0.13	0.000309
chr15 [§]	<i>MMP7</i>	6390211	6390212	+	8.57	6.29	0.19	0.000205
chr15 ^{†‡‡}	<i>PHOX2A</i>	52625075	52625076	–	6.29	6.14	0.17	0.0002289
chr19 [†]	<i>ITGA3</i>	37230331	37230332	+	12.29	11.00	0.12	0.0000896
chr21 ^{†§}	<i>DIO3</i>	68152290	68152291	+	14.86	14.14	0.18	0.0001115
chr22 ^{†‡}	<i>IL17RD</i>	44515500	44515501	–	18.57	18.14	0.27	0.00007418
chr23 ^{†‡}	<i>MDGA1</i>	11586464	11586465	–	21.14	15.43	0.17	0.0004023
chr23 ^{†‡}	<i>PHACTR1</i>	43380907	43380908	+	10.14	9.29	0.11	0.0002705
chr26 [†]	<i>ZWINT</i>	2851210	2851211	+	10.00	11.71	0.15	0.0001167
chr28 [§]	<i>C28H10orf35</i>	26104046	26104047	+	14.00	8.57	0.15	0.0003049
chr29 ^{†§}	<i>NRXN2</i>	43477994	43477995	+	10.57	6.71	0.28	0.0001266

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CpG island.

[‡]DNA methylation was exclusively contained in the gene body region of the gene.

^{‡‡}DNA methylation was located within an intron region.

[§]DNA methylation was located within an exon region.

Among these was a CHH site within the promoter region of the Immediate Early Response 2 (*IER2*) gene. The *IER2* gene encodes the Immediate Early Response 2 gene and is involved in neural development. Prenatal exposure to arsenic has been associated with altered *IER2* gene expression, with upregulation of *IER2* gene expression potentially serving as a biomarker of prenatal arsenic exposure (Fry et al., 2007).

The 8 significantly ($P \leq 0.05$) hypomethylated CHH sites located within promoter regions in PNS compared with Control calves are listed in Table 13. Among these was a CHH site within the promoter region of the Interferon Induced Transmembrane Protein 1/Interferon Induced Transmembrane Protein 2 (*IFITM1/IFITM2*) genes. Patients with

schizophrenia had increased *IFITM1* and *IFITM2* gene expression, which was likely indicative of an early environmental insult (Arion et al., 2007; Hwang et al., 2013).

Gene body regions. There were 133 differentially methylated CHH sites within gene body regions in PNS compared with Control bull calves (Table 1). A slightly greater percentage of these CHH sites were hypermethylated compared to hypomethylated (Table 1).

The top (lowest *P* values) 30 of 71 significantly ($P \leq 0.05$) hypermethylated CHH sites located within gene body regions in PNS compared with Control calves are listed in Table 14. Among these was a CHH site within the gene body region of the

Table 7. Top (lowest *P* values) 30 hypomethylated CpG sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr2 [§]	<i>CRYBA2</i>	107655452	107655453	+	17.86	12.57	-0.28	0.0001131
chr2 ^{†§}	<i>TMEM200B</i>	125010636	125010637	+	9.00	7.14	-0.12	6.12E-08
chr2 ^{**}	<i>DPP10</i>	68059420	68059421	+	13.43	10.43	-0.29	0.00007136
chr3 [§]	<i>LRRC42</i>	92815815	92815816	-	15.57	11.71	-0.12	0.0003827
chr3 ^{**}	<i>GRIK3</i>	109641283	109641284	-	13.86	9.43	-0.15	0.00006294
chr5 [§]	<i>FMNL3</i>	30363597	30363598	+	10.14	13.00	-0.16	0.00001178
chr6 ^{†*}	<i>NSG1</i>	106743364	106743365	-	11.14	8.71	-0.18	0.0001679
chr7 [‡]	<i>FDX1L</i>	16072432	16072433	+	9.43	9.57	-0.18	0.0004641
chr9 [§]	<i>OPRM1</i>	92152196	92152197	+	8.14	9.43	-0.13	0.0002767
chr11 ^{†*}	<i>GBGT1</i>	103182975	103182976	+	9.43	9.43	-0.6	0.0001948
chr11 ^{†*}	<i>NR5A1</i>	95535022	95535023	-	12.57	11.57	-0.12	0.00006977
chr13 ^{**}	<i>CUBN</i>	31602752	31602753	-	24.86	31.14	-0.1	0.0002177
chr13 ^{**}	<i>TGIF2</i>	66340155	66340156	+	8.86	10.14	-0.11	0.0003646
chr14 ^{†*}	<i>AGO2</i>	4114895	4114896	-	7.00	6.14	-0.43	0.000509
chr14 ^{**}	<i>CPQ</i>	69484191	69484192	+	9.86	9.71	-0.1	0.000005467
chr15 [§]	<i>APLNR</i>	81738036	81738037	+	25.57	24.57	-0.23	0.0004384
chr16 ^{**}	<i>ACOT7</i>	47839343	47839344	+	6.43	7.00	-0.11	0.0001594
chr16 ^{**}	<i>RGS7</i>	36591986	36591987	-	23.86	20.29	-0.13	0.000143
chr17 ^{**}	<i>SELM</i>	72065070	72065071	-	16.14	18.14	-0.27	0.0003458
chr18 ^{**}	<i>CBLC</i>	52956154	52956155	+	8.86	7.86	-0.2	0.0002327
chr19 [§]	<i>ABR</i>	22423681	22423682	-	13.71	14.00	-0.64	0.00003286
chr19 [‡]	<i>ERBB2</i>	40722503	40722504	+	13.14	9.57	-0.1	0.0004212
chr19 ^{†*}	<i>PMP22</i>	33382476	33382477	-	11.43	12.57	-0.12	0.00007101
chr22 ^{**}	<i>ALDH1L1</i>	61235727	61235728	+	17.00	12.57	-0.15	0.0000172
chr22 ^{**}	<i>LTF</i>	53523469	53523470	-	11.14	8.14	-0.24	0.0003724
chr24 ^{†*}	<i>ADCYAP1</i>	36118731	36118732	+	13.29	10.43	-0.16	0.0004943
chr26 [§]	<i>PRLHR</i>	39220962	39220963	+	11.43	9.29	-0.22	0.00008747
chr26 [‡]	<i>GLRX3</i>	49743379	49743380	+	12.43	9.57	-0.14	0.00004049
chr27 ^{**}	<i>MTUS1</i>	18657599	18657600	-	10.57	11.71	-0.21	0.00008578
chr29 ^{†*}	<i>MOB2</i>	50986147	50986148	-	13.86	11.29	-0.26	0.0003578

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CpG island.

^{*}DNA methylation was exclusively contained in the gene body region of the gene.

[‡]DNA methylation was located within an intron region.

[§]DNA methylation was located within an exon region.

Table 8. Hypermethylated CHG sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr1	<i>COLQ</i>	154285093	154285094	+	9.92	7.71	0.27	0.02224
chr12 [†]	<i>FAM155A</i>	87425041	87425042	+	8.00	5.71	0.16	0.000134
chr17 [*]	<i>CRYBB3</i>	67543284	67543285	+	12.43	12.86	0.17	0.02543

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CPG island.

^{*}DNA methylation was exclusively located within the promoter region.

Peroxisome Proliferator Activated Receptor Delta (*PPARD*) gene. The *PPARD* gene plays a key role in glucose and lipid metabolism (Brunmair et al., 2006). Male and female rats whose dams were

administered dexamethasone between 13 d of gestation and birth exhibited hyperinsulinemia, altered glucose and fatty acid metabolism, and females (only) had increased *PPARD* gene expression in

Table 9. Hypomethylated CHG sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	P value
chr4 [†]	<i>AKR1B1</i>	99033535	99033536	+	5.50	9.00	-0.1	0.00876
chr7*	<i>F2RL3</i>	6100112	6100113	-	9.13	13.14	-0.13	0.04596
chr17	<i>HIC2</i>	74182640	74182641	+	7.15	8.00	-0.11	0.01282
chr18	<i>CYP2S1</i>	50656104	50656105	+	9.63	7.43	-0.12	0.04467
chr21	<i>FURIN</i>	22213152	22213153	-	13.28	14.71	-0.11	0.04053
chr22	<i>QARS</i>	51484825	51484826	-	9.30	8.86	-0.13	0.01024
chr25**	<i>SBDS</i>	28636530	28636531	+	9.39	10.00	-0.11	0.01421

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CPG island.

*DNA methylation was exclusively located within the promoter region

Table 10. Top (lowest *P* values) 30 hypermethylated CHG sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	P value
chr1 [‡]	<i>COLQ</i>	154285093	154285094	+	11.29	7.71	0.27	0.02224
chr2**	<i>CAPZB</i>	133916622	133916623	+	10.57	8.57	0.1	0.03793
chr4 [§]	<i>ADCY1</i>	76872462	76872463	-	9.43	6.43	0.11	0.01105
chr4**	<i>LRGUK</i>	98721906	98721907	+	29.29	29.43	0.16	0.007548
chr4**	<i>LRGUK</i>	98750277	98750278	+	11.57	14.57	0.17	0.03429
chr4**	<i>SSPO</i>	113439057	113439058	+	8.29	5.00	0.11	0.04102
chr5**	<i>CNTN1</i>	40172096	40172097	-	5.43	5.57	0.11	0.01133
chr5**	<i>BTBD11</i>	71077326	71077327	+	11.14	13.86	0.13	0.01252
chr5**	<i>BTBD11</i>	71077307	71077308	+	12.71	15.29	0.12	0.01786
chr5**	<i>BTBD11</i>	71077336	71077337	+	9.71	11.86	0.14	0.0197
chr5**	<i>BTBD11</i>	71077331	71077332	+	10.57	13.43	0.14	0.0197
chr5**	<i>GTSE1</i>	117731307	117731308	+	14.14	13.71	0.12	0.02663
chr10**	<i>SLC8A3</i>	82134282	82134283	+	8.14	6.57	0.11	0.008943
chr11*	<i>ASS1</i>	100828396	100828397	-	8.14	9.14	0.37	0.01106
chr11**§	<i>GBGT1</i>	103182822	103182823	+	7.14	6.71	0.45	0.003044
chr12 [§]	<i>FAM155A</i>	87425041	87425042	+	9.14	5.71	0.16	0.000134
chr13**	<i>PLCB1</i>	1664698	1664699	+	9.14	7.71	0.39	0.02851
chr14**	<i>TRAPPC9</i>	4363947	4363948	+	9.86	7.57	0.26	0.03318
chr15**	<i>GDPD5</i>	55422374	55422375	+	9.29	7.00	0.2	0.002641
chr16**	<i>RGS7</i>	36590606	36590607	+	16.86	22.71	0.11	0.03004
chr16**	<i>RGS7</i>	36590615	36590616	+	10.43	11.86	0.1	0.03718
chr18 [§]	<i>MYADM</i>	62023403	62023404	-	11.00	6.14	0.18	0.04267
chr19**	<i>PRKCA</i>	63490629	63490630	+	9.57	10.71	0.1	0.02086
chr19**	<i>TRPV2</i>	33822975	33822976	-	8.57	5.86	0.12	0.01706
chr22 [§]	<i>RNF123</i>	51074315	51074316	-	9.14	6.00	0.15	0.01264
chr22 [§]	<i>RNF123</i>	51074297	51074298	-	9.14	6.00	0.12	0.03985
chr23**	<i>EFHC1</i>	24617468	24617469	+	9.71	7.71	0.11	0.01582
chr23**	<i>GMDS</i>	51389933	51389934	+	10.14	12.29	0.14	0.0292
chr25 [§]	<i>INTS1</i>	41995171	41995172	+	8.86	6.00	0.12	0.01351
chr27**	<i>CSGALNACT1</i>	37970135	37970136	+	10.86	8.29	0.42	0.01612

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CpG island.

*DNA methylation was exclusively contained in the gene body region of the gene.

[‡]DNA methylation was located within an intron region.

[§]DNA methylation was located within an exon region.

Table 11. Top (lowest *P* values) 30 hypomethylated CHG sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr1 ^{**}	<i>OXNAD1</i>	155073206	155073207	—	13.71	14.29	−0.34	0.01431
chr3 ^{**}	<i>TRAF3IP1</i>	118294302	118294303	—	7.57	6.57	−0.35	0.02739
chr4 [§]	<i>AKR1B1</i>	99033535	99033536	+	6.29	9.00	−0.1	0.00876
chr6 [§]	<i>PDGFRA</i>	71409734	71409735	+	12.57	8.29	−0.1	0.00002454
chr7 ^{**}	<i>NMRK2</i>	21292863	21292864	—	9.14	7.71	−0.35	0.001535
chr11 [§]	<i>IFITM5</i>	107203536	107203537	+	10.00	8.57	−0.17	0.03473
chr11 ^{**}	<i>FAM102A</i>	98633234	98633235	+	12.29	9.57	−0.22	0.01901
chr11 ^{**}	<i>GTF3C5</i>	103098035	103098036	—	8.43	7.29	−0.11	0.03774
chr11 ^{**}	<i>POMT1</i>	101661140	101661141	+	8.71	7.71	−0.45	0.005054
chr13 ^{**}	<i>CTNNBL1</i>	67382273	67382274	+	11.43	9.14	−0.11	0.01673
chr14 ^{**}	<i>DGAT1</i>	1799370	1799371	+	5.86	6.57	−0.11	0.01688
chr14 ^{**}	<i>DPYS</i>	62412347	62412348	+	10.57	11.43	−0.12	0.01354
chr16 ^{†§}	<i>CAMK1G</i>	75614071	75614072	+	7.86	7.43	−0.39	0.006132
chr17 ^{**}	<i>ISCU, LOC533308</i>	66689455	66689456	+	7.57	8.14	−0.11	0.01066
chr17 [‡]	<i>PRODH</i>	74182640	74182641	+	8.14	8.00	−0.11	0.01282
chr18 [§]	<i>GAN</i>	8018228	8018229	+	7.43	8.14	−0.1	0.008568
chr18 ^{†**}	<i>DDX19A</i>	1774349	1774350	+	9.29	7.14	−0.35	0.03018
chr18 ^{**}	<i>VAT1L</i>	5066203	5066204	—	9.29	7.43	−0.27	0.03996
chr19 ^{**}	<i>BAIAP2</i>	52216891	52216892	—	6.14	6.00	−0.1	0.01496
chr19 ^{**}	<i>EXOC7</i>	56206159	56206160	+	7.29	5.71	−0.11	0.02192
chr19 ^{**}	<i>SAMD14</i>	37166191	37166192	+	6.43	5.86	−0.1	0.01496
chr21 [‡]	<i>FURIN</i>	22213152	22213153	—	15.14	14.71	−0.11	0.04053
chr21 ^{**}	<i>LOC524810</i>	71546924	71546925	—	33.14	18.57	−0.12	0.02952
chr21 ^{**}	<i>OTUD7A</i>	30644587	30644588	+	12.71	10.00	−0.43	0.02269
chr22 ^{**}	<i>PTPRG</i>	39397678	39397679	—	10.00	9.00	−0.31	0.0106
chr22 [‡]	<i>QARS</i>	51484825	51484826	—	10.57	8.86	−0.13	0.01024
chr27 ^{†**}	<i>MTMR7</i>	18983825	18983826	—	7.29	9.86	−0.3	0.03781
chr27 ^{**}	<i>RNF122</i>	28663566	28663567	—	15.86	18.00	−0.24	0.007464
chr29 [§]	<i>TSSC4</i>	49837555	49837556	+	11.14	10.14	−0.15	0.003493
chr29 ^{**}	<i>FAT3</i>	2565099	2565100	+	16.29	11.86	−0.41	0.01684

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CpG island.

^{*}DNA methylation was exclusively contained in the gene body region of the gene.

[‡]DNA methylation was located within an intron region.

[§]DNA methylation was located within an exon region.

Table 12. Hypermethylated CHH sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr1 [*]	<i>C1H21orf91</i>	18719042	18719043	—	7.00	7.43	0.13	0.04541
chr1 [*]	<i>C1H21orf91</i>	18719029	18719030	—	7.00	7.43	0.1	0.01395
chr4 ^{**}	<i>ZYX</i>	107597956	107597957	—	5.13	6.57	0.19	0.01628
chr5	<i>LOC511240</i>	72050111	72050112	+	11.00	9.86	0.22	0.03802
chr5 [*]	<i>POLR3B</i>	70062294	70062295	—	15.50	14.14	0.11	0.03267
chr7 ^{**}	<i>IER2</i>	13545518	13545519	—	7.50	6.57	0.1	0.02712

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CPG island.

^{*}DNA methylation was exclusively located within the promoter region.

Table 13. Hypomethylated CHH sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	P value
chr4	<i>CPA5</i>	94878884	94878885	+	8.54	11.29	-0.14	0.03917
chr5*	<i>SRGAP1</i>	50119329	50119330	-	9.28	11.29	-0.12	0.0108
chr11	<i>IFITM2, IFITM1</i>	107192312	107192313	+	12.05	10.00	-0.22	0.006774
chr11	<i>IFITM2, IFITM1</i>	107192386	107192387	+	12.93	11.00	-0.19	0.01444
chr13*	<i>ZSWIM1</i>	75359296	75359297	-	7.73	8.57	-0.2	0.003527
chr15*	<i>MAML2</i>	14155574	14155575	+	6.77	7.14	-0.15	0.01866
chr15	<i>C15H11orf74</i>	67844693	67844694	+	10.75	13.43	-0.1	0.0426
chr18	<i>POP4</i>	40371128	40371129	-	10.53	14.29	-0.11	0.007739

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

*DNA methylation was located within a CPG island.

*DNA methylation was exclusively located within the promoter region.

skeletal muscle (Wyrwoll et al., 2008). Holstein cows fed a moderate-energy (1.47 Mcal/kg) diet compared to Controls (1.24 Mcal/kg) during late gestation had lower *PPARD* expression compared to Controls after parturition (Osorio et al., 2013).

The top (lowest *P* values) 30 of 62 significantly ($P \leq 0.05$) hypomethylated CHH sites located within gene body regions in PNS compared with Control calves are listed in Table 15. Among these was a CHH site within the gene body region of the Dihydropyrimidinase-like 2 (*DPYSL2*) gene. The *DPYSL2* gene is a collapsin response mediator protein that is involved in neurodevelopment, neurotransmission, and neurodegenerative diseases (Charrier et al., 2003). Rats whose dams underwent gestational stressors (i.e., restraint stress, food deprivation, forced swimming, reversed light-dark cycles, and overcrowding stress during dark cycles) between 14 d of gestation and birth exhibited decreased *DPYSL2* expression and potentially increased susceptibility to schizophrenic characteristics (Lee et al., 2015). Furthermore, rats whose dams were stressed by placing them on an elevated platform made of Plexiglass twice each day for a 10-min period between 12 and 16 d of gestation resulted in genome-wide alterations in gene expression, including expression of *DPYSL2* (Mychasiuk et al., 2011).

Canonical Pathways Altered in PNS Compared with Control Bull Calves

There were 113 canonical pathways altered ($P \leq 0.05$) in PNS compared with Control bull calves. Those signaling pathways and the differentially methylated genes in each pathway are represented in Supplementary Table S1. Among the pathways altered in PNS bull calves were pathways related to

behavior, stress response, immune function, metabolism, and cell signaling.

Pathways related to behavior, stress response, and neural function. Many genes and multiple canonical pathways related to behavior, stress response, and neural function were significantly altered in PNS compared with Control bull calves. Several of these pathways involved the hypothalamic-pituitary-adrenal (HPA) axis, neurotransmitter signaling, and opioid signaling. The “Corticotropin Releasing Hormone Signaling” pathway was activated in PNS bull calves (Supplementary Table S1). Other studies have reported differences in methylation of genes involved in HPA axis regulation, especially at the level of the glucocorticoid receptor gene, *NR3C1* (Perroud et al., 2014). Although this study did not show differences in *NR3C1* methylation, it did show differences at other levels of the HPA axis, including POMC methylation. “Dopamine-DARPP32 Feedback in cAMP Signaling and Dopamine Receptor Signaling” pathways were activated in PNS bull calves (Supplementary Table S1). Alterations in methylation of genes and canonical pathways related to behavior, stress response, and neural function agree with increased HPA axis activity and more excitable temperaments observed in calves in the larger population from which bull calves in this study were derived (Littlejohn et al., 2016). Previous reports suggest an influence of prenatal stress on dopamine regulation, especially at the level of COMT (Thompson et al., 2012) and dopamine receptors (Berger et al., 2002). The *COMT* gene and 2 dopamine receptor gene subtypes (*DRD1* and *DRD5*) were differentially methylated in PNS bull calves. The “GABA Receptor Signaling” pathway was altered

Table 14. Top (lowest *P* values) 30 hypermethylated CHH sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr1**	<i>COLQ</i>	154250800	154250801	–	14.00	10.71	0.31	0.02325
chr1**	<i>SH3BP5</i>	154100975	154100976	–	6.43	5.43	0.55	0.006741
chr3**	<i>ATG16L1</i>	113620590	113620591	–	12.71	12.00	0.14	0.001883
chr5**	<i>BTBD11</i>	71077321	71077322	+	12.00	14.14	0.14	0.006485
chr5**	<i>BTBD11</i>	71076725	71076726	–	26.00	17.14	0.11	0.009977
chr5**	<i>BTBD11</i>	71077305	71077306	+	12.71	15.29	0.14	0.01081
chr5**	<i>BTBD11</i>	71077300	71077301	+	11.57	13.57	0.13	0.01306
chr5**	<i>BTBD11</i>	71077320	71077321	+	13.00	15.29	0.11	0.01355
chr5**	<i>BTBD11</i>	71077302	71077303	+	12.29	15.00	0.12	0.01688
chr5**	<i>BTBD11</i>	71077322	71077323	+	12.00	14.29	0.13	0.01713
chr5**	<i>BTBD11</i>	71077304	71077305	+	12.86	15.14	0.12	0.01713
chr5**	<i>BTBD11</i>	71077313	71077314	+	12.86	15.14	0.12	0.01786
chr6**	<i>PDE5A</i>	7031186	7031187	–	13.57	9.71	0.25	0.00821
chr8**	<i>MOB3B</i>	16811946	16811947	+	9.57	11.14	0.35	0.01953
chr13**	<i>PKIG</i>	73718134	73718135	+	6.57	7.57	0.13	0.002584
chr13**	<i>PKIG</i>	73718143	73718144	+	6.57	7.57	0.13	0.003265
chr13**	<i>PKIG</i>	73718119	73718120	+	6.43	7.43	0.11	0.01428
chr19*	<i>SEZ6</i>	20882874	20882875	+	11.00	6.86	0.41	0.02207
chr19*	<i>NXN</i>	22530169	22530170	–	10.29	9.86	0.39	0.005695
chr20*	<i>DAP</i>	62637623	62637624	+	15.71	12.14	0.14	0.02114
chr20*	<i>SKIV2L2</i>	23806050	23806051	–	14.00	15.00	0.14	0.00286
chr21*	<i>FAN1</i>	27938912	27938913	–	10.71	9.29	0.29	0.01662
chr23*	<i>PHACTR1</i>	43337982	43337983	–	10.86	11.00	0.23	0.02088
chr23*	<i>PPARD</i>	9321494	9321495	+	7.86	8.57	0.11	0.01898
chr23*	<i>PRIM2</i>	2729219	2729220	–	5.86	9.71	0.15	0.02263
chr25*	<i>INTS1</i>	41995205	41995206	+	8.86	6.00	0.12	0.01351
chr25**	<i>LFNG</i>	41308254	41308255	–	13.29	12.00	0.17	0.01471
chr26*	<i>RBM20</i>	31655067	31655068	–	11.71	8.43	0.12	0.008981
chr27*	<i>RNF122</i>	28624366	28624367	+	29.00	14.86	0.11	0.01688
chr29*	<i>RCOR2</i>	42870766	42870767	+	11.00	9.57	0.1	0.01591

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

*DNA methylation was located within a CpG island.

**DNA methylation was exclusively contained in the gene body region of the gene.

†DNA methylation was located within an intron region.

§DNA methylation was located within an exon region.

in PNS bull calves (Supplementary Table S1). Other studies have reported differences in genes involved in GABA regulation, development of GABAergic cells, and associated anxious behavior (Berger et al., 2002; Lussier and Stevens, 2016). The “Serotonin Receptor Signaling” pathway, with an emphasis on serotonin receptor subtypes, was altered in PNS bull calves (Supplementary Table S1). Prenatal stress has been associated with differences in serotonin receptor binding, serotonin synthesis, and associated behavioral alterations (Peters, 1986; Van den Hove et al., 2006). Richetto et al. (2017) reported “Neuronal Differentiation” to be the most enriched gene ontology term associated with cell differentiation in mice that were exposed to a prenatal viral challenge on gestational

day 9 or 17. Significant subterms of “Neuronal Differentiation” included: “Gamma-Aminobutyric Acidergic Differentiation, Central Nervous System Differentiation, Noradrenergic System Differentiation, and Dopamine Differentiation.” Alterations to neurotransmitter pathways, such as dopamine, GABA, and serotonin have been associated with psychiatric disorders such as depression, anxiety, psychosis, and schizophrenia (Markham and Koenig, 2011). Furthermore, SNPs within the genes *POMC*, *DRD2*, *DRD3*, *HTR2A*, and *SLC18A2* have been associated with temperament in cattle (Garza-Brenner et al., 2017). These genes are part of the HPA, dopamine, and serotonin signaling pathways, each of which were predicted to be altered due to prenatal stress in this study. Previous

Table 15. Top (lowest *P* values) 30 hypomethylated CHH sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control calves¹

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	<i>P</i> value
chr1 ^{**}	<i>TBC1D5</i>	156103383	156103384	+	12.71	11.43	-0.27	0.02232
chr2 ^{**}	<i>UBR4</i>	134135665	134135666	+	9.29	7.86	-0.37	0.02694
chr3 ^{**}	<i>BARHL2</i>	52695313	52695314	-	15.43	12.57	-0.15	0.01694
chr7 ^{**}	<i>MAP2K2</i>	21141408	21141409	-	23.00	16.86	-0.29	0.02658
chr7 ^{**}	<i>PLVAP</i>	5687715	5687716	-	12.43	8.00	-0.12	0.0146
chr7 ^{**}	<i>SLC12A2</i>	27042120	27042121	+	9.00	10.57	-0.11	0.005449
chr8 ^{**}	<i>DPYSL2</i>	75136546	75136547	+	7.14	8.43	-0.32	0.03065
chr8 ^{**}	<i>GSN</i>	112604634	112604635	+	7.86	5.86	-0.11	0.02649
chr8 ^{**}	<i>TNFRSF10D</i>	71053599	71053600	+	72.00	44.71	-0.21	0.03105
chr10 ^{**}	<i>LRRIC16B</i>	20910389	20910390	+	14.00	11.71	-0.12	0.01559
chr11 ^{**}	<i>FUBP3</i>	100947908	100947909	+	11.43	13.71	-0.56	0.001667
chr11 [‡]	<i>IFITM2, IFITM1</i>	107192312	107192313	+	13.71	10.00	-0.22	0.006774
chr11 [‡]	<i>IFITM2, IFITM1</i>	107192386	107192387	+	14.71	11.00	-0.19	0.01444
chr11 ^{**}	<i>NACC2</i>	103605839	103605840	-	20.71	12.29	-0.49	0.006619
chr11 ^{**}	<i>NEK6</i>	95337824	95337825	+	12.86	9.57	-0.13	0.01493
chr12 ^{**}	<i>FARP1</i>	79248800	79248801	-	10.29	11.43	-0.28	0.02768
chr13 ^{**}	<i>BCAS1</i>	82210449	82210450	+	7.00	7.29	-0.24	0.03362
chr14 ^{**}	<i>ASAP1</i>	11452197	11452198	+	12.86	9.71	-0.13	0.02803
chr14 ^{**}	<i>DPYS</i>	62412342	62412343	+	10.43	11.43	-0.12	0.01296
chr14 ^{**}	<i>DPYS</i>	62412355	62412356	+	10.43	11.29	-0.12	0.01296
chr14 ^{**}	<i>DPYS</i>	62412345	62412346	+	10.57	11.43	-0.12	0.01354
chr15 ^{**}	<i>LOC509058</i>	76786259	76786260	-	10.86	8.57	-0.43	0.01323
chr18 [§]	<i>POP4</i>	40371128	40371129	-	12.00	14.29	-0.11	0.007739
chr18 ^{**}	<i>MTHFSD</i>	12404197	12404198	-	10.14	9.29	-0.12	0.03374
chr18 ^{**}	<i>PEPD</i>	44033725	44033726	-	6.86	7.29	-0.5	0.0007633
chr19 ^{**}	<i>RAB37</i>	57344842	57344843	-	17.43	13.71	-0.11	0.01538
chr21 ^{**}	<i>AKAP6</i>	43500188	43500189	+	14.86	12.00	-0.24	0.02095
chr21 ^{**}	<i>LOC524810</i>	71577837	71577838	-	19.86	16.29	-0.17	0.02587
chr21 ^{**}	<i>PSTPIP1</i>	32652323	32652324	-	6.86	6.86	-0.24	0.01042
chr27 ^{**}	<i>KCNU1</i>	31979794	31979795	+	8.71	8.57	-0.27	0.03064

¹In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[‡]DNA methylation was located within a CpG island.

^{*}DNA methylation was exclusively contained in the gene body region of the gene.

[‡]DNA methylation was located within an intron region.

[§]DNA methylation was located within an exon region.

studies are consistent with alterations in predicted pathways related to behavior, stress response, and neural function in PNS bull calves. Immune cells and brain cells may exhibit similarities in differential methylation induced by prenatal or life experiences (Provençal et al., 2012; Tylee et al., 2013; Massart et al., 2016a; Seifuddin et al., 2017). For example, rhesus monkeys that were reared by an inanimate surrogate and age-matched peers rather than their biological dam exhibited significant overlap of differential methylation of DNA in T cells and cells from the prefrontal cortex (Provençal et al., 2012). Furthermore, 9 mo after a peripheral nerve injury (i.e., a chronic pain model) was induced in young rats, 72% of the promoters that were differentially methylated in T-cells were also differentially

methylated in cells from the prefrontal cortex (Massart et al., 2016a). WBC have been reported to serve as an acceptable surrogate to reflect differential methylation in brain cells; however, differential methylation in WBC may only represent a portion of differential methylation in cells from neural tissues of interest.

Pathways related to immune function. Many genes and multiple canonical pathways related to immune function were significantly altered in PNS compared with Control bull calves. This might have been expected considering DNA methylation was assessed in WBC. Several of these key pathways included, “Leukocyte Extravasation Signaling, IL-15 Production, and IL-8 Signaling, Phagosome

Formation, and B Cell Activating Factor Signaling” (Supplementary Table S1). Alterations in methylation of immune function related genes and canonical pathways in PNS bull calves were related to differences in cytokine concentrations and leukocyte counts in response to an endotoxin challenge (Littlejohn et al., 2018). Specifically, PNS bulls had a larger increase in IFN- γ from basal concentrations and a larger decrease in circulating monocyte counts suggesting increased extravasation of monocytes. These observations can be directly related to some of the altered pathways in this study. Alterations in immune function due to prenatal stress have been evidenced by hematology and cytokine alterations in primates, rodents, and swine (Reyes and Coe, 1997; Vanbesien-Mailliot et al., 2007; Couret et al., 2009). Richetto et al. (2017) reported the “Leukocyte Differentiation” process to be significantly altered in mice that were prenatally exposed to a viral challenge on gestational day 9 or 17. Cao-Lei et al. (2014) reported that children whose mothers were in the 1998 ice storm in Quebec during gestation had altered genome-wide DNA methylation in T cells at 13 yr of age. Six of the top 10 functions that were reported to be altered in those PNS children were related to immune function. Specifically, each of those functions was directly involved in T lymphocyte function (Cao-Lei et al., 2014). Previous studies as well as methylomic and phenotypic differences related to immune function in PNS bull calves suggest a potential influence of prenatal stress on overall health and immune response in bovine.

Pathways related to metabolism. Multiple genes and canonical pathways related to metabolic function were significantly altered in PNS compared with Control bull calves. Several of these pathways included, “Leptin Signaling in Obesity, Adipogenesis pathway, and Glycine Cleavage Complex” (Supplementary Table S1). Alterations in methylation of genes and canonical pathways that were related to metabolic function were linked to differences in metabolic function observed in a subset of the larger population of bulls from which bulls in this study were derived (d’Orey Branco et al., 2016). These alterations included a differential insulin response to a glucose challenge. Specifically, PNS bulls took less time to reach peak insulin response to glucose administration, decreased time to return to baseline, and a smaller area under the insulin response curve compared to Controls. This suggested PNS bulls to have an increased sensitivity to insulin (d’Orey Branco et al., 2016). Cao-Lei et al. (2014) reported the influence of the prenatal Quebec

ice storm stressor on “Type 1 Diabetes Mellitus Signaling,” as predicted by differential DNA methylation of T cells at 13 yr of age. Furthermore, male mice whose dams were exposed to repeated exposure to an aggressive lactating female during late gestation had increased circulating triglyceride concentrations, decreased hepatic 5 α -reductase, decreased Ppara mRNA expression, and decreased subcutaneous fat PEPCCK mRNA expression (Brunton et al., 2013). Alterations to metabolism related pathways have implications for metabolic diseases, such as diabetes. However, alterations to metabolic processes in cattle might translate to differences in growth, gain, or feed efficiency, which could result in profitability differences in PNS cattle.

Pathways related to cell pluripotency and signaling. Many genes and canonical pathways related to cell pluripotency and cell signaling were significantly altered in PNS compared with Control bull calves.

Two pathways related to cell pluripotency that were predicted by IPA to be altered in PNS bulls were also predicted to be altered in rhesus monkeys that experienced early life maternal separation stress and a lack of maternal-rearing, including: “Human Embryonic Stem Cell Pluripotency and Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency (Massart et al., 2016b).” Because DNA methylation is a primary regulator of cell-specific functions (Razin and Riggs, 1980; Suelves et al., 2016), predicted alterations to pluripotency of cells is logical and suggests developmental programming of various cell types.

Among altered cell signaling pathways was, “cAMP-mediated signaling, G-Protein Coupled Receptor Signaling, Gas Signaling, Phospholipase C Signaling, Tec Kinase Signaling, Gai Signaling, and TGF- β Signaling” (Supplementary Table S1). Cao-Lei et al. (2014) also reported an influence of the prenatal Quebec ice storm stressor on “Phospholipase C Signaling,” as predicted by differential DNA methylation of T cells at 13 yr of age. Massart et al. (2016b) reported that rhesus monkeys undergoing early life maternal separation stress and a lack of maternal-rearing had altered genome-wide DNA methylation in CD3+ T cells from day 14 to 2 y of age. From that study, 5 of the top 6 canonical pathways (assessed by IPA) altered in PNS monkeys were also significantly differentially methylated in PNS bull calves in this study. Two of those mutually altered pathways were related to cell signaling: “G-protein coupled receptor signaling and cAMP-mediated cell signaling.” Alterations to cell signaling pathways in previous studies and in PNS

bull calves, suggests a potential influence of prenatal stress on many biological processes through cell signaling processes employed across many cell types.

Physiological Functions Altered in PNS Compared with Control Bull Calves

The top (most enriched) 10 “Physiological System Development and Function” terms generated by IPA software (Fig. 3) suggest a broad influence of prenatal stress on physiological systems. Six of the top 10 terms were related to developmental processes, which could occur during prenatal programming of physiological systems in utero. It is consistent with this laboratory’s previous phenotypic findings (Littlejohn et al., 2016) that “Behavior” and “Nervous System Development and Function” were within the top 10 most enriched terms (Fig. 3). These data suggest a broad influence of prenatal stress on prenatal development of physiological systems.

Genome-wide Overview of Differentially Methylated Regions

Overall, genome-wide distribution of differential DNA methylation (hypermethylation and hypomethylation) in PNS compared with Control bull calves were similar to previous reports in humans and nonhuman primates (Cao-Lei et al., 2014; Massart et al., 2016b). Heat maps in Figs. 4 and 5 compare the most significant (lowest *P* values) 100 methylation ratios (specific to each individual animal) that were hypermethylated and hypomethylated, respectively, in PNS compared with Control bull calves. Hierarchical cluster analysis was performed with regard to prenatal treatment. The

dendrograms above and to the left of the heat maps represent this clustering.

Genome-wide chromosome distribution of differentially methylated CpG sites with regard to significance, $-\log_{10}(\text{p-value})$, is represented in the form of a Manhattan plot in Fig. 6. Fourteen CpG sites surpassed the $-\log_{10}(\text{p-value})$ threshold of 5. Of these sites, 1 was located exclusively within a promoter region (*EIF3J*), 1 was located within a promoter and gene body region (*CLSTN2*), 2 were located exclusively within a gene body region (*TMEM200B* and *CPQ*), and 10 were located within an intergenic region. These data reveal a diverse distribution of differentially methylated CpG sites in PNS calves, suggesting a substantial influence of prenatal environment on gene function. Genome-wide chromosomal distributions of DNA methylation ratios are represented in Fig. 7; these ratios were calculated from CpG sites within all region types with a minimum sequence read depth of 5 times ($P \leq 1.0$). Although there was a greater percentage of significantly hypomethylated than hypermethylated CpG sites in PNS compared with Control bull calves (Table 1), the genome-wide illustration of all analyzed CpG sites in PNS and Control calves suggests the opposite relationship (Fig. 7). Overall, these data show genome-wide distribution of differential methylation across each chromosome in PNS compared with Control bull calves.

Methylation of DNA acts to control gene activity and is a primary regulator of cell-specific functions (Razin and Riggs, 1980). Methylation status of DNA can be influenced by external stimuli, resulting in altered gene expression and phenotype (Feinberg, 2010; Szyf, 2012). The epigenome is most sensitive to change during embryogenesis and perinatal development (Reik, 2007). Early life

Figure 3. Top (most enriched) 10 “Physiological System Development and Function” terms associated with alterations to methylation of DNA in prenatally stressed bull calves (generated by IPA software).

Figure 4. Comparison of the top 100 methylation ratios that were hypermethylated in prenatally stressed (PNS) compared with Control bull calves (1.0=Greatest degree of methylation; 0.0=Least degree of methylation).

Figure 5. Comparison of the top 100 methylation ratios that were hypomethylated in prenatally stressed (PNS) compared with Control bull calves (1.0=Greatest degree of methylation; 0.0=Least degree of methylation).

development affects many biological mechanisms, which shape phenotype in beef cattle (Alford et al., 2007; Brickell et al., 2009). Therefore, the objective of this study was evaluation of the influence of a prenatal transportation stressor on the postnatal epigenome, and how PNS-induced alterations to the epigenome might affect biological systems, and thereby economically relevant traits in cattle. In the

current study, methylation was assessed in DNA of WBC from PNS and Control bull calves. Prenatal and early life stressors have been reported to alter DNA methylation in various leukocyte types (Provençal et al., 2012; Cao-Lei et al., 2014). It is important to acknowledge that differential methylation has been reported among leukocyte types (Adalsteinsson et al., 2012), which raises concern

Figure 6. Manhattan plot of $-\log_{10}(p\text{-values})$ for all differentially methylated CpG sites across the genome (all region types).

Figure 7. Comparison of genome-wide prenatally stressed (PNS; red line) and Control (blue line) CpG site methylation ratios (all region types).

with assessing a fundamentally heterogeneous population of WBC. However, [Heiss and Brenner \(2017\)](#) reported relatively low variation in DNA methylation among leukocyte types in humans, suggesting WBC could be an acceptable population of cells for DNA methylation analysis. Changes in methylation of DNA in WBC induced by prenatal or life experiences have been reported to be correlated with changes in methylation of DNA in various brain tissues ([Provençal et al., 2012](#), [Tylee et al., 2013](#); [Massart et al., 2016a](#); [Seifuddin et al., 2017](#)); therefore, methylation of DNA from WBC can provide insight to methylation in other tissues such as neural tissue. Because differential methylation in WBC may only represent a portion of differential methylation in cells from neural tissues of interest, greater insight might be achieved by assessing tissue-specific methylation. It is also important to understand the influence of changes in DNA methylation on the transcriptome and resultant phenotype. Therefore, future studies should assess tissue-specific differences in methylation of DNA and its association with differences in transcription and phenotype due to prenatal stress.

CONCLUSIONS

To our knowledge, these data are the first reports of a genome-wide assessment of DNA methylation in PNS calves. Overall, these data exhibited similarities with data from models of prenatal stress in humans and nonhuman primates ([Cao-Lei et al., 2014](#); [Massart et al., 2016b](#)). Prenatal transportation stress in cattle altered genome-wide DNA methylation profiles, which were predicted by IPA to alter canonical pathways related to behavior, stress response, neural function, immune function, metabolism, cell signaling, and other biological processes. Alterations in behavior, stress response, metabolism, and immune function are related with phenotypic differences observed in the population of calves from which bull calves in this study were derived ([d'Orey Branco, et al., 2016](#); [Littlejohn et al., 2016; 2018](#)). The data presented herein demonstrate alterations of the methylome in PNS calves. Future evaluation of the inter-relationships of the methylome and transcriptome in specific tissues with phenotype of PNS calves will increase the understanding of the impact of prenatal stress on

economically and biologically relevant phenotypic traits in a bovine model.

SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Animal Science* online.

LITERATURE CITED

- Adalsteinsson, B. T., H. Gudnason, T. Aspelund, T. B. Harris, L. J. Launer, G. Eiriksdottir, A. V. Smith, and V. Gudnason. 2012. Heterogeneity in white blood cells has potential to confound DNA methylation measurements. *Plos One* 7:e46705. doi:10.1371/journal.pone.0046705
- Alford, A., L. Cafe, P. Greenwood, and G. Griffith. 2007. The economic consequences of early-life nutritional constraints in crossbred cattle bred on the NSW North Coast. Economic Research Report No. 33. NSW Department of Primary Industries, Armidale, May.
- Andreou, D., E. Söderman, T. Axelsson, G. C. Sedvall, L. Terenius, I. Agartz, and E. G. Jönsson. 2016. Associations between a locus downstream DRD1 gene and cerebrospinal fluid dopamine metabolite concentrations in psychosis. *Neurosci. Lett.* 619:126–130. doi:10.1016/j.neulet.2016.03.005
- Arion, D., T. Unger, D. A. Lewis, P. Levitt, and K. Mirnics. 2007. Molecular evidence for increased expression of genes related to immune and chaperone function in the prefrontal cortex in schizophrenia. *Biol. Psychiatry* 62:711–721. doi:10.1016/j.biopsych.2006.12.021
- Barua, S., S. Kuizon, K. K. Chadman, M. J. Flory, W. T. Brown, and M. A. Junaid. 2014. Single-base resolution of mouse offspring brain methylome reveals epigenome modifications caused by gestational folic acid. *Epigenetics Chromatin* 7:3. doi:10.1186/1756-8935-7-3
- Benediktsson, R., A. A. Calder, C. R. Edwards, and J. R. Seckl. 1997. Placental 11 beta-hydroxysteroid dehydrogenase: a key regulator of fetal glucocorticoid exposure. *Clin. Endocrinol. (Oxf)*. 46:161–166. doi:10.1046/j.1365-2265.1997.1230939.x
- Berger, M. A., V. G. Barros, M. I. Sarchi, F. I. Tarazi, and M. C. Antonelli. 2002. Long-term effects of prenatal stress on dopamine and glutamate receptors in adult rat brain. *Neurochem. Res.* 27:1525–1533. doi:10.1023/A:1021656607278
- Bernal, J. 2005. Thyroid hormones and brain development. *Vitam. Horm.* 71:95–122. doi:10.1016/S0083-6729(05)71004-9
- Breitling, L. P., K. Salzmann, D. Rothenbacher, B. Burwinkel, and H. Brenner. 2012. Smoking, F2RL3 methylation, and prognosis in stable coronary heart disease. *Eur. Heart J.* 33:2841–2848. doi:10.1093/eurheartj/ehs091
- Brickell, J. S., M. M. McGowan, and D. C. Wathes. 2009. Effect of management factors and blood metabolites during the rearing period on growth in dairy heifers on UK farms. *Domest. Anim. Endocrinol.* 36:67–81. doi:10.1016/j.domaniend.2008.10.005
- Brunmair, B., K. Staniek, J. Dörig, Z. Szöcs, K. Stadlbauer, V. Marian, F. Gras, C. Anderwald, H. Nohl, W. Waldhäusl, et al. 2006. Activation of PPAR-delta in isolated rat skeletal muscle switches fuel preference from glucose to fatty acids. *Diabetologia* 49:2713–2722. doi:10.1007/s00125-006-0357-6
- Brunton, P. J., K. M. Sullivan, D. Kerrigan, J. A. Russell, J. R. Seckl, and A. J. Drake. 2013. Sex-specific effects of prenatal stress on glucose homeostasis and peripheral metabolism in rats. *J. Endocrinol.* 217:161–173. doi:10.1530/JOE-12-0540
- Cao-Lei, L., R. Massart, M. J. Suderman, Z. Machnes, G. Elgbeili, D. P. Laplante, M. Szyf, and S. King. 2014. DNA methylation signatures triggered by prenatal maternal stress exposure to a natural disaster: project ice storm. *Plos One* 9:e107653. doi:10.1371/journal.pone.0107653
- Charrier, E., S. Reibel, V. Rogemond, M. Agüera, N. Thomasset, and J. Honnorat. 2003. Collapsin response mediator proteins (CRMPs): involvement in nervous system development and adult neurodegenerative disorders. *Mol. Neurobiol.* 28:51–64. doi:10.1385/MN:28:1:51
- Chocyk, A., A. Przyborowska, D. Dudys, I. Majcher, M. Maćkowiak, and K. Wędzony. 2011. The impact of maternal separation on the number of tyrosine hydroxylase-expressing midbrain neurons during different stages of ontogenesis. *Neuroscience* 182:43–61. doi:10.1016/j.neuroscience.2011.03.008
- Clarke, A. S., A. Soto, T. Bergholz, and M. L. Schneider. 1996. Maternal gestational stress alters adaptive and social behavior in adolescent Rhesus monkey offspring. *Infant Behav. Dev.* 19:451–461. doi:10.1016/S0163-6383(96)90006-5
- Couret, D., A. Prunier, A. M. Mounier, F. Thomas, I. P. Oswald, and E. Merlot. 2009. Comparative effects of a prenatal stress occurring during early or late gestation on pig immune response. *Physiol. Behav.* 98:498–504. doi:10.1016/j.physbeh.2009.08.003
- d'Orey Branco, R. A., D. A. Neuendorff, S. E. Schmidt, N. C. Burdick Sanchez, J. A. Carroll, T. H. Welsh, R. D. Randel. 2016. Influence of prenatal stress on insulin response to a glucose challenge in yearling Brahman bulls. *J. Anim. Sci.* 94(Suppl 1):33. (Abstr. 066). doi:10.2527/ssasas2015-066
- Ehrlich, M., M. A. Gama-Sosa, L. H. Huang, R. M. Midgett, K. C. Kuo, R. A. McCune, and C. Gehrke. 1982. Amount and distribution of 5-methylcytosine in human DNA from different types of tissues or cells. *Nucleic Acids Res.* 10:2709–2721. doi:10.1093/nar/10.8.2709
- FASS. 2010. Guide for the care and use of agricultural animals in research and teaching. 3rd ed. FASS, Champaign, IL.
- Feinberg, A. P. 2010. Genome-scale approaches to the epigenetics of common human disease. *Virchows Arch.* 456:13–21. doi:10.1007/s00428-009-0847-2
- Fry, R. C., P. Navasumrit, C. Valiathan, J. P. Svensson, B. J. Hogan, M. Luo, S. Bhattacharya, K. Kandjanapa, S. Soontararuks, S. Nookabkaew, et al. 2007. Activation of inflammation/NF-kappaB signaling in infants born to arsenic-exposed mothers. *Plos Genet.* 3:e207. doi:10.1371/journal.pgen.0030207
- Garbett, K. A., E. Y. Hsiao, S. Kálmán, P. H. Patterson, and K. Mirnics. 2012. Effects of maternal immune activation on gene expression patterns in the fetal brain. *Transl. Psychiatry* 2:e98. doi:10.1038/tp.2012.24
- Garza-Brenner, E., A. M. Sifuentes-Rincón, R. D. Randel, F. A. Paredes-Sánchez, G. M. Parra-Bracamonte, W. Arellano Vera, F. A. Rodríguez Almeida, and A. Segura Cabrera. 2017. Association of SNPs in dopamine and serotonin pathway genes and their interacting genes with

- temperament traits in Charolais cows. *J. Appl. Genet.* 58:363–371. doi:10.1007/s13353-016-0383-0
- Harada, S., H. Tachikawa, and Y. Kawanishi. 2003. A possible association between an insertion/deletion polymorphism of the NQO2 gene and schizophrenia. *Psychiatr. Genet.* 13:205–209. doi:10.1097/01.ypg.0000071601.59979.47
- Heiss, J. A., and H. Brenner. 2017. Impact of confounding by leukocyte composition on associations of leukocyte DNA methylation with common risk factors. *Epigenomics* 9:659–668. doi:10.2217/epi-2016-0154
- Hellman, A., and A. Chess. 2007. Gene body-specific methylation on the active X chromosome. *Science* 315:1141–1143. doi:10.1126/science.1136352
- Hwang, Y., J. Kim, J. Y. Shin, J. I. Kim, J. S. Seo, M. J. Webster, D. Lee, and S. Kim. 2013. Gene expression profiling by mRNA sequencing reveals increased expression of immune/inflammation-related genes in the hippocampus of individuals with schizophrenia. *Transl. Psychiatry* 3:e321. doi:10.1038/tp.2013.94
- Ishihama, T., Y. Ago, N. Shintani, H. Hashimoto, A. Baba, K. Takuma, and T. Matsuda. 2010. Environmental factors during early developmental period influence psychobehavioral abnormalities in adult PACAP-deficient mice. *Behav. Brain Res.* 209:274–280. doi:10.1016/j.bbr.2010.02.009
- Lay, D. C., Jr, R. D. Randel, T. H. Friend, O. C. Jenkins, D. A. Neuendorff, D. M. Bushong, E. K. Lanier, and M. K. Bjorge. 1997. Effects of prenatal stress on suckling calves. *J. Anim. Sci.* 75:3143–3151. doi:10.2527/1997.75123143x
- Lee, S., T. Devamani, H. D. Song, M. Sandhu, A. Larsen, R. Sommesse, A. Jain, N. Vaidehi, and S. Sivaramakrishnan. 2017. Distinct structural mechanisms determine substrate affinity and kinase activity of protein kinase C. *J. Biol. Chem.* 292:16300–16309. doi:10.1074/jbc.M117.804781
- Lee, H., J. Joo, S. S. Nah, J. W. Kim, H. K. Kim, J. T. Kwon, H. Y. Lee, Y. O. Kim, and H. J. Kim. 2015. Changes in dpysl2 expression are associated with prenatally stressed rat offspring and susceptibility to schizophrenia in humans. *Int. J. Mol. Med.* 35:1574–1586. doi:10.3892/ijmm.2015.2161
- Levine, A., G. L. Cantoni, and A. Razin. 1991. Inhibition of promoter activity by methylation: possible involvement of protein mediators. *Proc. Natl. Acad. Sci. USA.* 88:6515–6518. doi:10.1073/pnas.88.15.6515
- Lister, R., M. Pelizzola, R. H. Dowen, R. D. Hawkins, G. Hon, J. Tonti-Filippini, J. R. Nery, L. Lee, Z. Ye, Q. M. Ngo, et al. 2009. Human DNA methylomes at base resolution show widespread epigenomic differences. *Nature* 462:315–322. doi:10.1038/nature08514
- Littlejohn, B. P., D. M. Price, J. P. Banta, A. W. Lewis, D. A. Neuendorff, J. A. Carroll, R. C. Vann, T. H. Welsh, and R. D. Randel. 2016. Prenatal transportation stress alters temperament and serum cortisol concentrations in suckling Brahman calves. *J. Anim. Sci.* 94:602–609. doi:10.2527/jas.2015-9635
- Littlejohn, B. P., N. C. Burdick Sanchez, J. A. Carroll, D. M. Price, R. C. Vann, T. H. Welsh, Jr., and R. D. Randel. 2018. Influence of prenatal transportation stress on innate immune response to an endotoxin challenge in weaned Brahman bull calves. *Stress* doi:10.1080/10253890.2018.1523895
- Lussier, S. J., and H. E. Stevens. 2016. Delays in GABAergic interneuron development and behavioral inhibition after prenatal stress. *Dev. Neurobiol.* 76:1078–1091. doi:10.1002/dneu.22376
- Maeyama, H., T. Hirasawa, Y. Tahara, C. Obata, H. Kasai, K. Moriishi, K. Mochizuki, and T. Kubota. 2015. Maternal restraint stress during pregnancy in mice induces 11 β -HSD1-associated metabolic changes in the livers of the offspring. *J. Dev. Orig. Health Dis.* 6:105–114. doi:10.1017/S2040174415000100
- Markham, J. A., and J. I. Koenig. 2011. Prenatal stress: role in psychotic and depressive diseases. *Psychopharmacology (Berl)*. 214:89–106. doi:10.1007/s00213-010-2035-0
- Masilamoni, J. G., E. P. Jesudason, B. Baben, C. E. Jebaraj, S. Dhandayuthapani, and R. Jayakumar. 2006. Molecular chaperone alpha-crystallin prevents detrimental effects of neuroinflammation. *Biochim. Biophys. Acta* 1762:284–293. doi:10.1016/j.bbadis.2005.11.007
- Massart, R., S. Dymov, M. Millecamps, M. Suderman, S. Gregoire, K. Koenigs, S. Alvarado, M. Tajerian, L. S. Stone, and M. Szyf. 2016a. Overlapping signatures of chronic pain in the DNA methylation landscape of prefrontal cortex and peripheral T cells. *Sci. Rep.* 6:19615. doi:10.1038/srep19615
- Massart, R., Z. Nemoda, M. J. Suderman, S. Sutti, A. M. Ruggiero, A. M. Dettmer, S. J. Suomi, and M. Szyf. 2016b. Early life adversity alters normal sex-dependent developmental dynamics of DNA methylation. *Dev. Psychopathol.* 28(4pt2):1259–1272. doi:10.1017/S0954579416000833
- Middleton, F. A., K. Mirnics, J. N. Pierri, D. A. Lewis, and P. Levitt. 2002. Gene expression profiling reveals alterations of specific metabolic pathways in schizophrenia. *J. Neurosci.* 22:2718–2729. doi:20026209
- Mohammed, S. A., S. A. Rahamtalla, S. S. Ahmed, A. Elhafiz, B. M. Dousa, K. M. Elamin, and M. K. A. Ahmed. 2015. DGAT1 gene in dairy cattle. *GJASR.* 3:191–198.
- Mueller, B. R., and T. L. Bale. 2008. Sex-specific programming of offspring emotionality after stress early in pregnancy. *J. Neurosci.* 28:9055–9065. doi:10.1523/JNEUROSCI.1424-08.2008
- Mychasiuk, R., R. Gibb, and B. Kolb. 2011. Prenatal stress produces sexually dimorphic and regionally specific changes in gene expression in hippocampus and frontal cortex of developing rat offspring. *Dev. Neurosci.* 33:531–538. doi:10.1159/000335524
- Osorio, J. S., E. Trevisi, M. A. Ballou, G. Bertoni, J. K. Drackley, and J. J. Looor. 2013. Effect of the level of maternal energy intake prepartum on immunometabolic markers, polymorphonuclear leukocyte function, and neutrophil gene network expression in neonatal Holstein heifer calves. *J. Dairy Sci.* 96:3573–3587. doi:10.3168/jds.2012–5759
- Ousman, S. S., B. H. Tomooka, J. M. van Noort, E. F. Wawrousek, K. C. O'Connor, D. A. Hafler, R. A. Sobel, W. H. Robinson, and L. Steinman. 2007. Protective and therapeutic role for alphas-crystallin in autoimmune demyelination. *Nature* 448:474–479. doi:10.1038/nature05935
- Perroud, N., E. Rutembesa, A. Paoloni-Giacobino, J. Mutabaruka, L. Mutesa, L. Stenz, A. Malafosse, and F. Karege. 2014. The tutsi genocide and transgenerational transmission of maternal stress: epigenetics and biology of the HPA axis. *World J. Biol. Psychiatry* 15:334–345. doi:10.3109/15622975.2013.866693
- Peters, D. A. 1986. Prenatal stress: effect on development of rat brain serotonergic neurons. *Pharmacol. Biochem. Behav.* 24:1377–1382. doi:10.1016/0091-3057(86)90198-X
- Plagge, A., E. Gordon, W. Dean, R. Boiani, S. Cinti, J. Peters, and G. Kelsey. 2004. The imprinted signaling protein XL

- alpha s is required for postnatal adaptation to feeding. *Nat. Genet.* 36:818–826. doi:10.1038/ng1397
- Price, D. M., A. W. Lewis, D. A. Neuendorff, J. A. Carroll, N. C. Burdick Sanchez, R. C. Vann, T. H. Welsh, and R. D. Randel. 2015. Physiological and metabolic responses of gestating Brahman cows to repeated transportation. *J. Anim. Sci.* 93:737–745. doi:10.2527/jas.2013-7508
- Provençal, N., M. J. Suderman, C. Guillemin, R. Massart, A. Ruggiero, D. Wang, A. J. Bennett, P. J. Pierre, D. P. Friedman, S. M. Côté, et al. 2012. The signature of maternal rearing in the methylome in rhesus macaque prefrontal cortex and T cells. *J. Neurosci.* 32:15626–15642. doi:10.1523/JNEUROSCI.1470-12.2012
- de Quervain, D. J., I. T. Kolassa, S. Ackermann, A. Aerni, P. Boesiger, P. Demougin, T. Elbert, V. Ertl, L. Gschwind, N. Hadziselimovic, et al. 2012. Pkca is genetically linked to memory capacity in healthy subjects and to risk for posttraumatic stress disorder in genocide survivors. *Proc. Natl. Acad. Sci. USA.* 109:8746–8751. doi:10.1073/pnas.1200857109
- Ramsahoye, B. H., D. Binizskiewicz, F. Lyko, V. Clark, A. P. Bird, and R. Jaenisch. 2000. Non-CpG methylation is prevalent in embryonic stem cells and may be mediated by DNA methyltransferase 3a. *Proc. Natl. Acad. Sci. USA.* 97:5237–5242. doi:10.1073/pnas.97.10.5237
- Razin, A., and A. D. Riggs. 1980. DNA methylation and gene function. *Science* 210:604–610. doi:10.1126/science.6254144
- Reik, W. 2007. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature* 447:425–432. doi:10.1038/nature05918
- Ressler, K. J., K. B. Mercer, B. Bradley, T. Jovanovic, A. Mahan, K. Kerley, S. D. Norrholm, V. Kilaru, A. K. Smith, A. J. Myers, et al. 2011. Post-traumatic stress disorder is associated with PACAP and the PAC1 receptor. *Nature* 470:492–497. doi:10.1038/nature09856
- Reyes, T. M., and C. L. Coe. 1997. Prenatal manipulations reduce the proinflammatory response to a cytokine challenge in juvenile monkeys. *Brain Res.* 769:29–35. doi:10.1016/S0006-8993(97)00687-2
- Richetto, J., R. Massart, U. Weber-Stadlbauer, M. Szyf, M. A. Riva, and U. Meyer. 2017. Genome-wide DNA methylation changes in a mouse model of infection-mediated neurodevelopmental disorders. *Biol. Psychiatry* 81:265–276. doi:10.1016/j.biopsych.2016.08.010
- Rodgers, A. B., and T. L. Bale. 2015. Germ cell origins of posttraumatic stress disorder risk: the transgenerational impact of parental stress experience. *Biol. Psychiatry* 78:307–314. doi:10.1016/j.biopsych.2015.03.018
- Rodgers, A. B., C. P. Morgan, S. L. Bronson, S. Revello, and T. L. Bale. 2013. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. *J. Neurosci.* 33:9003–9012. doi:10.1523/JNEUROSCI.0914-13.2013
- Seifuddin, F., G. Wand, O. Cox, M. Pirooznia, L. Moody, X. Yang, J. Tai, G. Boersma, K. Tamashiro, P. Zandi, et al. 2017. Genome-wide methyl-seq analysis of blood-brain targets of glucocorticoid exposure. *Epigenetics* 12:637–652. doi:10.1080/15592294.2017.1334025
- Shinohara, H., Y. Inaguma, S. Goto, T. Inagaki, and K. Kato. 1993. Alpha Bcrystallin and HSP28 are enhanced in the cerebral cortex of patients with Alzheimer's disease. *J. Neurol. Sci.* 119:203–208. doi:10.1016/0022-510X(93)90135-L
- Shirane, K., H. Toh, H. Kobayashi, F. Miura, H. Chiba, T. Ito, T. Kono, and H. Sasaki. 2013. Mouse oocyte methylomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA methyltransferases. *Plos Genet.* 9:e1003439. doi:10.1371/journal.pgen.1003439
- Shukla, P. K., L. J. Sittig, T. M. Ullmann, and E. E. Redei. 2011. Candidate placental biomarkers for intrauterine alcohol exposure. *Alcohol. Clin. Exp. Res.* 35:559–565. doi:10.1111/j.1530-0277.2010.01373.x
- Sikora, K. M., D. A. Magee, E. W. Berkowicz, D. P. Berry, D. J. Howard, M. P. Mullen, R. D. Evans, D. E. Machugh, and C. Spillane. 2011. DNA sequence polymorphisms within the bovine guanine nucleotide-binding protein Gs subunit alpha (Gsa)-encoding (GNAS) genomic imprinting domain are associated with performance traits. *BMC Genet.* 12:4. doi:10.1186/1471-2156-12-4
- Silva, C. F., E. S. Sartorelli, A. C. Castilho, R. A. Satrapa, R. Z. Puelker, E. M. Razza, J. S. Ticianelli, H. P. Eduardo, B. Loureiro, and C. M. Barros. 2013. Effects of heat stress on development, quality and survival of bos indicus and bos taurus embryos produced in vitro. *Theriogenology* 79:351–357. doi:10.1016/j.theriogenology.2012.10.003
- Stirrat, L. I., B. G. Sengers, J. E. Norman, N. Z. M. Homer, R. Andrew, R. M. Lewis, and R. M. Reynolds. 2018. Transfer and metabolism of cortisol by the isolated perfused human placenta. *J. Clin. Endocrinol. Metab.* 103:640–648. doi:10.1210/je.2017-02140
- Stohn, J. P., M. E. Martinez, M. Zafer, D. López-Espíndola, L. M. Keyes, and A. Hernandez. 2018. Increased aggression and lack of maternal behavior in dio3-deficient mice are associated with abnormalities in oxytocin and vasopressin systems. *Genes. Brain. Behav.* 17:23–35. doi:10.1111/gbb.12400
- Suelves, M., E. Carrió, Y. Núñez-Álvarez, and M. A. Peinado. 2016. DNA methylation dynamics in cellular commitment and differentiation. *Brief. Funct. Genomics* 15:443–453. doi:10.1093/bfgp/ewl017
- Szyf, M. 2012. The early-life social environment and DNA methylation. *Clin. Genet.* 81:341–349. doi:10.1111/j.1399-0004.2012.01843.x
- Tate, P. H., and A. P. Bird. 1993. Effects of DNA methylation on DNA-binding proteins and gene expression. *Curr. Opin. Genet. Dev.* 3:226–231. doi:10.1016/0959-437X(93)90027-M
- Thompson, J. M., E. J. Sonuga-Barke, A. R. Morgan, C. M. Cornforth, D. Turic, L. R. Ferguson, E. A. Mitchell, and K. E. Waldie. 2012. The catechol-O-methyltransferase (COMT) Val158Met polymorphism moderates the effect of antenatal stress on childhood behavioural problems: longitudinal evidence across multiple ages. *Dev. Med. Child Neurol.* 54:148–154. doi:10.1111/j.1469-8749.2011.04129.x
- Tsai, C. E., S. P. Lin, M. Ito, N. Takagi, S. Takada, and A. C. Ferguson-Smith. 2002. Genomic imprinting contributes to thyroid hormone metabolism in the mouse embryo. *Curr. Biol.* 12:1221–1226. doi:10.1016/S0960-9822(02)00951-X
- Tylee, D. S., D. M. Kawaguchi, and S. J. Glatt. 2013. On the outside, looking in: a review and evaluation of the comparability of blood and brain “-omes”. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 162B:595–603. doi:10.1002/ajmg.b.32150
- Van den Hove, D. L. A., J. M. Lauderc, A. Scheepensd, J. Prickaertsb, C. E. Blancao, and H. W. M. Steinbuschb.

2006. Prenatal stress in the rat alters 5-HT1A receptor binding in the ventral hippocampus. *Brain Res.* 1090:29–34. doi:10.1016/j.brainres.2006.03.057
- Vanbesien-Mailliot, C. C., I. Wolowczuk, J. Mairesse, O. Viltart, M. Delacre, J. Khalife, M. C. Chartier-Harlin, and S. Maccari. 2007. Prenatal stress has pro-inflammatory consequences on the immune system in adult rats. *Psychoneuroendocrinology* 32:114–124. doi:10.1016/j.psyneuen.2006.11.005
- Vangeel, E. B., B. Izzi, T. Hompes, K. Vansteelandt, D. Lambrechts, K. Freson, and S. Claes. 2015. DNA methylation in imprinted genes IGF2 and GNASXL is associated with prenatal maternal stress. *Genes. Brain. Behav.* 14:573–582. doi:10.1111/gbb.12249
- Varley, K. E., J. Gertz, K. M. Bowling, S. L. Parker, T. E. Reddy, F. Pauli-Behn, M. K. Cross, B. A. Williams, J. A. Stamatoyannopoulos, G. E. Crawford, et al. 2013. Dynamic DNA methylation across diverse human cell lines and tissues. *Genome Res.* 23:555–567. doi:10.1101/gr.147942.112
- Wyatt, G. R. 1950. Occurrence of 5-methylcytosine in nucleic acids. *Nature* 166:237–238. doi:10.1038/166237b0
- Wyrwoll, C. S., P. J. Mark, T. A. Mori, and B. J. Waddell. 2008. Developmental programming of adult hyperinsulinemia, increased proinflammatory cytokine production, and altered skeletal muscle expression of SLC2A4 (GLUT4) and uncoupling protein 3. *J. Endocrinol.* 198:571–579. doi:10.1677/JOE-08-0210
- Xie, Y., A. Awonuga, J. Liu, E. Rings, E. E. Puscheck, and D. A. Rappolee. 2013. Stress induces AMPK-dependent loss of potency factors *id2* and *cdx2* in early embryos and stem cells [corrected]. *Stem Cells Dev.* 22:1564–1575. doi:10.1089/scd.2012.0352
- Yang, W., D. Li, G. Wang, X. Wu, M. Zhang, C. Zhang, Y. Cui, and S. Li. 2017. Expression and imprinting of *DIO3* and *DIO3OS* genes in Holstein cattle. *J. Genet.* 96:333–339. doi:10.1007/s12041-017-0780-0
- Yen, C. L., S. J. Stone, S. Koliwad, C. Harris, and R. V. Farese, Jr. 2008. DGAT enzymes and triacylglycerol biosynthesis. *J. Lipid Res.* 49:2283–2301. doi:10.1194/jlr.R800018-JLR200
- Zhang, X. G., H. Zhang, X. L. Liang, Q. Liu, H. Y. Wang, B. Cao, J. Cao, S. Liu, Y. J. Long, W.Y. Xie, and D. Z. Peng. 2016. Epigenetic mechanism of maternal post-traumatic stress disorder in delayed rat offspring development: dysregulation of methylation and gene expression. *Genet. Mol. Res.* 15:gmr9009. doi:10.4238/gmr.15039009
- Zhou, Y., L. Xu, D. M. Bickhart, E. H. Abdel Hay, S. G. Schroeder, E. E. Connor, L. J. Alexander, T. S. Sonstegard, C. P. Van Tassell, H. Chen, et al. 2016. Reduced representation bisulphite sequencing of ten bovine somatic tissues reveals DNA methylation patterns and their impacts on gene expression. *BMC Genomics.* 17:779. doi:10.1186/s12864-016-3116-1
- Zhu, X., T. Li, S. Peng, X. Ma, X. Chen, and X. Zhang. 2010. Maternal deprivation-caused behavioral abnormalities in adult rats relate to a non-methylation-regulated D2 receptor levels in the nucleus accumbens. *Behav. Brain Res.* 209:281–288. doi:10.1016/j.bbr.2010.02.005
- Ziller, M. J., F. Müller, J. Liao, Y. Zhang, H. Gu, C. Bock, P. Boyle, C. B. Epstein, B. E. Bernstein, T. Lengauer, et al. 2011. Genomic distribution and inter-sample variation of non-CpG methylation across human cell types. *Plos Genet.* 7:e1002389. doi:10.1371/journal.pgen.1002389