Genomic Effect of Triclosan on the Fetal Hypothalamus: Evidence for Altered Neuropeptide Regulation

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Triclosan (TCS), an antibacterial compound commonly added to personal care products, could be an endocrine disruptor at low doses. Although TCS has been shown to alter fetal physiology, its effects in the developing fetal brain are unknown. We hypothesize that exposure to TCS during fetal life could affect fetal hypothalamic gene expression. The objective of this study was to use transcriptomics and systems analysis to identify significantly altered biological processes in the late gestation ovine fetal hypothalamus after direct or indirect exposure to low doses of TCS. For direct TCS exposure, chronically catheterized late gestation fetal sheep were infused with vehicle (n=4) or TCS (250 μg/day; n=4) iv. For indirect TCS exposure, TCS (100 μg/kg/day; n=3) or vehicle (n=3) was infused into the maternal circulation. Fetal hypothalami were collected after 2 days of infusion, and gene expression was measured through microarray. Hierarchical clustering of all samples according to gene expression profiles showed that samples from the TCS treated animals clustered apart from the controls. Gene Set Enrichment Analysis revealed that fetal hypothalamic genes stimulated by maternal and fetal TCS infusion were significantly enriching for cell cycle, reproductive process, and feeding behavior, while the inhibited genes were significantly enriching for chromatin modification and metabolism of steroids, lipoproteins, fatty acids, and glucose ($p<0.05$). In conclusion, short-term infusion of TCS induces vigorous changes in the fetal hypothalamic transcriptomics, which are mainly related to food intake pathways and metabolism. If these changes persist to postnatal life, they could result in adverse consequences in adulthood.

Triclosan (TCS: 5-chloro-2-(2,4-dichlorophenoxy)-phenol) is a synthetic antibacterial compound that is commonly added to personal care products (1). TCS acts as an antibacterial because it interrupts fatty acid synthesis (2) by inhibiting enoyl reductase in bacteria (3). Because of its action on a bacterial enzyme that – except for within the gastrointestinal (GI) microbiota (4)– is not expressed in the human being, it is thought to be safe for use in humans (5).

TCS specifically has been associated with fetal growth restriction. A recent epidemiologic study showed that maternal urinary TCS concentration was negatively correlated with fetal growth variables in late gestation (6). In rodents, TCS decreases circulating concentrations of thyroid hormones in the pregnant dam and fetus (7, 8), and prolonged exposure to TCS induced fetal growth restriction as well as delayed vaginal opening in the postnatal female offspring (9).

These studies have demonstrated that TCS exposure during fetal life affects fetal and postnatal physiology. Yet, the effect of TCS on the developing fetal brain remains unknown. We performed the present study to test whether TCS, in doses that are similar to exposure in humans from personal care products (10, 11), has an effect on the fetal

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Abbreviations:

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hypothalamus. The hypothalamus was selected because it plays a central role in the coordination of fetal autonomic and neuroendocrine function, including control of blood pressure (BP), responses to stress, and control of ingestive behavior postnatally (12). We used the ovine fetus as animal model since the entire gestational equivalent of human brain development occurs in utero (13), and the size of the sheep fetus allows us to analyze molecular responses in defined brain regions.

We have recently used transcriptomics and systems biology analysis to understand the response of the fetal hypothalamus to in utero stressors (14, 15). In the present study, we used this approach to identify the fetal hypothalamic response to TCS using two routes of administration (fetal intravenous (IV) or maternal IV). Using this broad transcriptomics approach, we tested the hypothesis that TCS alters fetal hypothalamic gene transcription, and that the pattern of changes in the transcriptomics will reveal molecular pathways and potentially neuropeptides or neuronal pathways that are susceptible to TCS in the late gestation fetal hypothalamus.

Materials and Methods

Chemicals

Triclosan, 99.8% pure, was purchased from TCI America (Philadelphia, PA) and recrystallized from ethanol:water before administering to sheep. All other chemicals were purchased from laboratory suppliers and were of reagent grade or better.

Animal procedures

All of these experiments were performed in accordance with the Guiding Principles for Research Involving Animals and Human Beings published by the American Physiological Society and were approved by the University of Florida Institutional Animal Care and Use Committee.

To test the fetal response to direct fetal IV infusion of TCS (n = 4) or DMSO:water::50:50 vehicle solution (n = 4), we used the chronically-catheterized fetal sheep model of in utero development and physiology. Time-dated pregnant ewes and their fetuses were both surgically prepared with indwelling vascular catheters (femoral arteries and veins, and in the fetuses, an additional catheter in the amniotic fluid). In a second study designed to test the fetal response to maternal IV infusion of TCS, time-dated pregnant ewes (but not their fetuses) were surgically prepared with catheters in femoral arteries and veins or in the external jugular vein to infuse TCS (n = 3) or DMSO vehicle (n = 3).

Food was withheld from the ewes for 24 hours before surgery (124 ± 3 days gestation). Anesthesia was induced by mask with isoflurane, then intubated and anesthesia was maintained using isoflurane (0.5%–2%) in oxygen. Surgery and postsurgical care were performed as previously described (16). A minimum of 5 days after surgery, we infused TCS at a rate of 0.1 mg/kg/d for 2 days. We estimated fetal body weight to be approximately 2.5 kg; as a result, we infused 0.25 mg/d into each fetus intravenously (IV) in a total volume of 10 ml/d. Body weights of the pregnant ewes in the second study ranged from 63 to 75 kg; therefore, we infused 6.3–7.5 mg/d IV into these ewes. The TCS dose is within the range of likely environmental exposure to TCS from plaque-reducing toothpaste, which contains 0.3% TCS and antibacterial soap, which contains 0.1–0.2% Triclosan, as measured in human samples (17).

Sample Collection

Blood samples were drawn (5 mL from fetus and ewe in the first study and 5 mL from ewe in the second study) before, and after 1 and 2 days of infusion. At the end of the infusion, we humanely sacrificed the ewes and fetuses with an overdose of sodium pentobarbital. In each study, fetal brains were rapidly removed, dissected into distinct regions, and snap frozen in liquid nitrogen. Tissues collected included the fetal hypothalamus, pituitary, hippocampus, cerebral cortex, medullary brainstem, and cerebellum. Tissues were stored at −80°C until processed for RNA. In the present study, only the RNA isolated from the hypothalamus was analyzed.

RNA Extraction and Preparation

Messenger RNA was extracted and purified as previously reported (15). RNA integrity (RIN) numbers of the purified mRNA samples was measured using an Agilent Bioanalyzer, and ranged from 7.4 to 8.7. DNase treatment, synthesis of cRNA, and labeling were performed as previously described (15). The resulting labeled cRNA was analyzed with the NanoDrop spectrophotometer, and the specific activities and the yields of the cRNAs ranged from 10.2 to 12.8 pmol Cy3/μg RNA and from 3.0 to 9.6 μg, respectively. The labeled cRNA was stored at −80°C until use. Microarray hybridization, washing, and scanning were performed as previously described (14). We used the Agilent-019 921 Sheep Gene Expression Microarray 8 × 15k, G4813A, that we had previously annotated (GEO accession number: GPL14112) (14).

Microarray data preparation

The limma package was employed to import the raw data into R (http://www.r-project.org), perform background correction and normalize the data using the quantile normalization method (18). Control probes and probes that were less than 10% brighter than the negative control probes were filtered out, and then the intensity of probes with the same probe identity were averaged. In order to eliminate multiple probes and facilitate the biological interpretation of the results, probes that were annotated with the same gene name were collapsed using the MaxMean function of the WGCNA package in R (19). This method assigns the highest intensity value as the expression value of the gene, based on the assumption that the probe with the highest intensity value had the best hybridization to the cDNA. After probe filtering and collapsing, the gene expression list contained 8196 unique Official Symbols for 8196 genes, out of 15 008 probes in the original platform. Microarray data have been deposited in the NCBI Gene Expression Omnibus under accession number GSE69275.

Unsupervised data analysis

An exploratory microarray data analysis was performed initially, to determine the relatedness between microarray samples

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according gene expression profiles. Microarray samples were: TCS infused into the fetus (TCSF, n = 4), TCS infused into the ewe (TCSM, n = 3), saline infused into the fetus (ControlF, n = 4), and saline infused into the ewe (ControlM, n = 3). Correlations between samples were evaluated by application of hierarchical clustering and principal component analysis (PCA) methods. For hierarchical clustering, we used the open software Cluster 3.0 (20). Data was adjusted by centering the gene expressions by the median. Gene expression and microarray samples were clustered according to centroid linkage, after similarity measurement by centered correlation. The principal components of the data were calculated with the function prcomp from the stats package for R (21). The first and second principal components were plotted in a 2-D plot using the plot function from the graphics package for R. The 14 data points were colored according their group.

Gene set enrichment analysis (GSEA):

This analytical method considers first the gene expression profiles from two classes to rank genes according to the strength of the correlation with a class. Then, given a priori defined sets of genes, the method will determine if genes (belonging to the same set) are randomly distributed throughout the ranked list or mainly found at the top or bottom, ie, significantly overexpressed in one or other class (22). For our study, we defined the gene sets as genes sharing the same biological process. A significant biological process was defined by a nominal p-value < 0.05, after 1000 permutations. GSEA (http://software.broadinstitute.org/gsea) is an open software developed at the Broad Institute of MIT and Harvard, that determines whether an a priori defined set of genes shows statistically differences between two biological states (22, 23). One limitation of this tool is that relies on biological information from previous experiments; for this reason, the ability of GSEA to identify processes relevant to fetal hypothalamic function might be underpowered.

Transcription factor analysis.

WebGestalt was used for detection of transcription factor binding sites that are statistically significantly over-represented among the differentially regulated genes. This analysis is based on an ORACLE relational database GeneKeyDB, which uses a strong gene and protein centric viewpoint (24).

Statistical analysis

The GSEA method finds significant biological processes in the data independently of statistical analysis. However, in order to corroborate the results we applied a moderated *t* test to find differentially expressed genes (DEG) in the data to then perform ontology analysis of them. The limma package was used for the moderated *t* test, applying the empirical Bayes method proposed by Smyth (25), which produces more stable estimates when the number of replicates is small. Genes were considered differentially expressed with p-values of < 0.05. Gene ontology analysis for the significant up or down-regulated genes was performed with the DAVID database (26), searching for enriched biological processes ($P < .05$) in the data.

Quantitative Real-Time (qRT)-PCR Validation

The RNA samples extracted from the fetal hypothalami of the four groups were converted to cDNA with a High Capacity cDNA Archive kit using the methodology recommended by the kit manufacturer (Applied Biosystems, Foster City, Calif., USA). The newly synthesized cDNA was stored at –20°C until qRT-PCR was performed. The following genes were selected for further validation by qRT-PCR: AGRP (agouti related protein), ASIP (Agouti signaling protein), POMC (propiomelanocortin), and FASN (fatty acid synthase).

Relative expression of selected genes was determined using primers (Sigma-Aldrich, St Louis, MO) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primers were designed with Primer Express software (Applied Biosystems, Foster City, CA) from the corresponding ovine mRNA. Primers sequences and accession numbers are reported in Supplemental Table 1. All primer pairs had efficiencies greater than 95%. The abundance of β-actin mRNA was determined in each sample, using primers and VIC Taqman probes designed from the ovine β-actin sequence and Taqman qRT-PCR master mix (Applied Biosystems, Foster City, CA). All samples were run in triplicate for each gene and for β-actin. There were no differences in β -actin expression among the groups. Relative mRNA expression of each gene was calculated by determining change in threshold cycle (ΔCt) between the mean Ct for each gene and the mean Ct for β -actin mRNA from the same sample. The effect of maternal or fetal TCS infusion on each gene was analyzed by ANOVA using the ΔCt values. Data were graphed as the mean fold change in expression relative to the respective control group; fold change in each sample was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta$Ct is the difference between ΔCt in each sample and the mean ΔCt in the control group. For all statistical analyses, the criterion for achieving statistical significance was $P < .05$.

Results

Unsupervised data analysis

The heat map resulting from the hierarchical clustering method and the principal components plot are shown in Figure 1. The heat map (Figure 1A) demonstrates that all samples belonging to TCSF are clustered together and they are similar to TCSM, except for one sample (TCSM 3) that clusters with ControlF 4 sample. The PCA (Figure 1b) corroborates the relatedness of the samples. Visually, control samples are closer to TCSM samples (although they are not mixed) while TCSF segregated apart from the other groups. These analyses suggest that TCS infusion induces a strong effect in fetal hypothalamic gene expression, and this effect is more pronounced if the TCS is infused directly into the fetus.

Gene set enrichment analysis (GSEA)

The GSEA analysis was performed selecting the biological processes (from gene ontology) as the a priori defined genes set dat. Comparisons were done between TCSM and ControlM samples and between TCSF and ControlF samples. Each gene set has a leading-edge subset, that is, the core of genes that accounts for the enrichment

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signal. If enriched biological processes were redundant, a leading edge analysis was performed. This analysis determines common genes in the leading-edge subsets between similar biological processes. For each group, the enriched biological processes and only the genes belonging to the leading edges are shown in Table 1.

Statistical Analysis

The total number of DEG was higher for the comparison between TCSF and ControlF samples than for TCSM and ControlM samples, as expected after the unsupervised analysis results (2740 and 1461 DEG, respectively, Supplemental Table 2 A-D). There were significant DEG overlaps between maternal and fetal TCS infusion for both upregulated and downregulated genes (p-value < 0.00001 by Pearson's χ^2 test, Figure 2). However, there were a much larger number of genes stimulated or inhibited only by maternal or fetal TCS infusion, as shown in Figure 2.

Ontology analysis

An extended number of biological processes were significantly enriched ($P < .05$) with the up or down regulated genes by maternal or fetal TCS infusion (Supplemental Table 3 A-D, respectively), that could be summarized in the following biological processes.

For TCSM, the more representative up-regulated biological processes were: mitosis, cell cycle, cell adhesion, female pregnancy, response to hormone stimulus, developmental maturation, calcium ion homeostasis, feeding behavior and regulation of inflammatory response and cytokine production (Supplemental Table 3A). Representative down-regulated biological processes were: nitrogen compound biosynthesis process, cellular homeostasis, adaptive and humoral immune response, response to hypoxia, leukocyte migration, generation of precursor metabolites and energy, protein maturation, response to steroid hormone stimulus, proteolysis, and monosaccharide metabolic process (Supplemental Table 3B).

For TCSF, representative up-regulated biological processes were: regulation of apoptosis, cell-cell signaling, female pregnancy, inflammatory response, regulation of cytokine production, homeostatic process, response to insulin stimulus and regulation of secretion (Supplemental Table 3C). Representative down-regulated biological processes were: chromatin/histone modification, actin filament-based process, in utero development, regulation of neuron differentiation, protein catabolic process, glucose catabolic process, cholesterol metabolic process and fatty acid metabolic process (Supplemental Table 3D).

The ontology analysis performed with the DEG, selected after the statistical analysis, shows that most of the enriched biological processes correspond with the findings of the GSEA, corroborating these results. Also, the ontol-

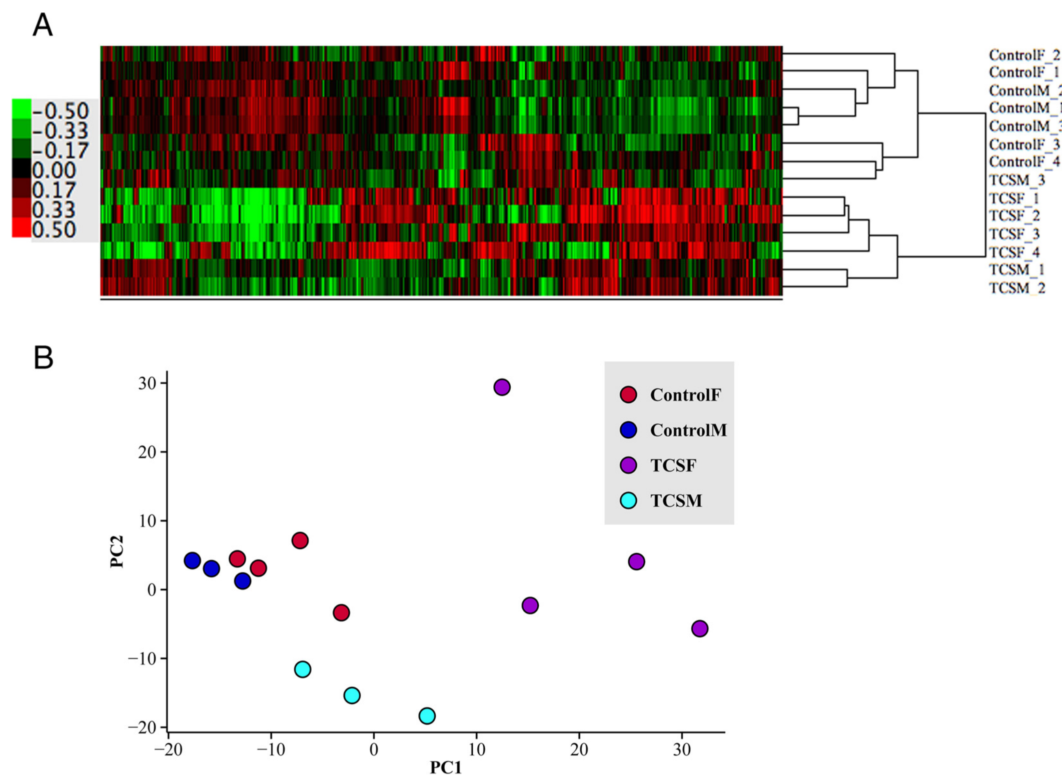

Figure 1. Unsupervised analysis of the microarray samples. The heat map (A) and the principal component (PC) plot (B) show that samples belonging to the same group tend to cluster together.

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Table 1. Enriched biological processes and core genes on fetal hypothalamic samples up-regulated or down-regulated by maternal or fetal infusion of Triclosan.

Biological Process	Core Genes	p -value
Up-regulated by maternal Triclosan infusion		
Mitotic cell cycle	KIF15 AURKA KIF2C SUGT1 FBXO5 NDC80 DLGAP5 BIRC5 CENPE ESPL1 MPHOSPH6 SMAD3 UBE2C TPX2 KIF11 CCNA2 BUB1 SKP2 MAD2L2 TTK NCAPH KNTC1 SMC4 CDC123 PLK1 CLIP1 KPNA2	<0.001
Cell-cell signaling	CGA GHRH TSHB TFAP2C POMC NPY ADM CALCA GNRH1 CCL5 TSHR PMP22 AREG EDN3 EFNA4 TBX3 GCH1 WISP1 CXCL5 SST KCNN3 PTHLH GHRL SYN2 MERTK PRG3 NQO1 GRM5 SH2D1A	<0.001
Female pregnancy	ADM TRO SFRP4 TAC3 FSHB OVGP1 SPRR2E PTHLH GHRL OXTR FLT1	<0.001
Cell adhesion	SAA1 CYTIP IL8 EMCN TGM2 IL12A ARHGDIB SPN	0.003
G Protein coupled receptor	AGRP GHRH TSHB NPY CALCA TAC3 TSHR PTAFR GCG GLP1R CD3E SST IL8 PTHLH GHRL CXCR5 SSTR5 CRHR1 ADRB1 GRM5 RGS14 CXCR6 CCR3 CCRL2 GHRHR PTGER1 UCN CXCR2 RGS1 PIK3CG APOC3 HCRTR1 ANG OPRM1 GPRC5C CXCL12 CXCR4 CCL2 HRH2 APOA1 AGTR1	0.003
Reproductive process	ADM TRO SFRP4 TAC3 ACE2 FSHB OVGP1 TOP2A SMAD3 TBX3 SPRR2E IL8 PTHLH GHRL OXTR CRHR1 IDE FLT1 CXCR6 MKKS	0.003
Behavior	AGRP SAA1 CCL28 NPY CCL5 PTAFR GCG CXCL5 GHRL IL8 AGTR2 BRS3 PMCH CCR3 CCRL2 CXCR2 SPN HCRTR1 OPRM1 CXCL12 CXCR4 LECT2 CCL2	0.005
Down-regulated by maternal Triclosan infusion		
Chromatin/ Histone modification	PRMT7 KDM4A PHB KAT5 SIRT2 UBE2N WHSC1L1 NSD1 RBM14	0.03
Protein processing	SCLY OAZ1 ASRGL1 NFS1 ADI1 WARS GCLM SLC7A5 HGD HPRT1 BBOX1 GLUD1 ALDH6A1 SLC7A4 SMS ATF4 MAT2B PEPD BCKDHA DHPS FPGS ALDH5A1 CCBL1 QDPR PAH BCAT1 DARS GOT1 PLOD1	0.03
Lipoprotein metabolic process	APOA2 SCARF1 FNTB PIGO DPM1 LDLR PPT1	0.04
Steroid metabolic Process	SCARB1 HSD17B11 STARD3 STUB1 CLN6 PPARD NPC1 APOL2 HSD17B6 IL4	0.05
Fatty acid metabolic process	ACADM ADIPOR1 ALDH5A1 CD74 CPT1A CROT ECHS1 FADS2 FASN HACL1 HADHB PPARD PTGES3	0.05
Up-regulated by fetal Triclosan infusion		
Cell-Cell Signaling	GHRH CXCL10 GNRH1 SH2D1A S100A9 MME PRG3 CALCA CCL4 CCL15 CCL5 IL18 GCHFR CHRNB1 TFAP2C GRM4 TNFRSF11A SST WISP1 SYPL1 GCH1 AREG C1QA SNAI2 POMC NPY MGST2 SYN2 ASIP KLF10 GRM5 CXCL13 BMP2 INHBA	0.005
Inflammatory response	S100A12 CXCL10 S100A9 ANXA1 CCL4 CCL5 HDAC4 IL1RAP AIF1 CYBB	0.006
Cytokine production	TLR1 PRG3 CALCA IL6 IL18 SRGN SMAD3 TLR3 INHBA EBI3 TLR6 HIF1A CEBPG APOA2 SMAD4 INHA ATP6AP2 SFTPD MALT1	
Regulation of secretion	GHRH ANG SRGN INHBA DNAJC1 APOA2 MYO6 INHA	0.01
Cellular homeostasis	F2RL1 CALCA CD55 CCL15 CCL5 MT2A ATP1A1 SAA1 CP GPR98	0.03
Behavior	CXCL10 AGRP CCL15 CCL5 SAA1 PMCH NPY BRS3 ACSL4 CCL2 PLAUR CCRL2 CXCL13	0.04
Down-regulated by fetal Triclosan infusion		
Chromatin/ Histone modification	HDAC6 CREBBP UBE2N NSD1 SET EHMT1 RBM14 KAT5 KDM4A SIRT2 PPARGC1A	0.01
Lipoprotein metabolic process	SCARF1 FNTB PPT1 PIGO LDLR	0.03
Central nervous system development	EIF2B1 TNC NNAT PROP1 UBE3A CTNS SMARCA1 EIF2B2 ROBO2 S100B BTD ARNT2 PITPNM1 DRP2 SLIT1 BPTF PAX6 GPR56 RNF103 SLIT3 ALDH5A1 NCOA6 NPAS2 SHH PPT1 NKX2–2	0.03
Gamete generation	FSHB CDYL PARN PICK1 USP9× TOB2 MAST2 HMGCR TESK1 SERPINA5 RUVBL1 DEDD BRD2	0.05

ogy analysis refined the results, since it demonstrated a wider range of significant biological processes.

Transcription factor analysis

Results of transcription factor analysis are reported in Table 2. Analysis transcription factor binding sites revealed that SP1, ELK1, LEF1, ERR1, ER (estrogen receptor alpha), GR (glucocorticoid receptor), T3R (thyroid hormone receptor), and isoforms of PPAR (peroxisome proliferator activated receptor) are significantly associated with genes up- and down-regulated by either maternal or fetal infusion of TCS. Genes upregulated by fetal TCS infusion but not altered by maternal infusions are associated with NFY (nuclear transcription factor Y) and GABP (GA binding protein alpha chain). Conversely, genes downregulated by fetal TCS infusion but not altered by maternal infusions are associated with PAX4 (paired box gene 4) and SREBP (Sterol regulatory element-binding protein). Other transcription factors were associated with responses in more than one group (eg, c-Myc, which is associated with both up- and down-regulated genes after fetal infusion and downregulated genes after maternal infusion).

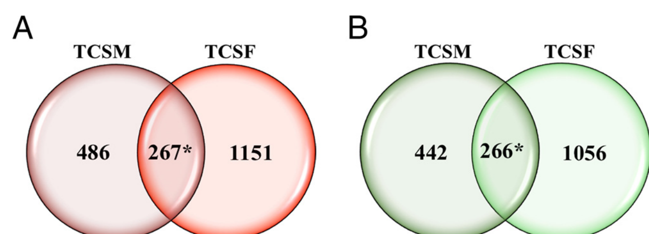

Figure 2. Venn Diagrams of Differentially Expressed Genes (DEG). The Venn Diagrams show significant overlap between DEG upregulated (A) or downregulated (B) by maternal (TCSM) and fetal Triclosan (TCSF) infusion (*p-value < 0.00001 by Pearson's χ^2 test).

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Table 2. Transcription factor binding sites significantly over-represented among genes up-regulated or down-regulated by maternal or fetal infusion of Triclosan.

	Transcription Factor	Site		Genes/Total	Adjusted probability
		Up-regulated by fetal Triclosan infusion			
1	SP1	hsa	GGGCGGR V$SP1 Q6	265/2891	9.87 e^{-57}
2	ELK1	hsa	SCGGAAGY V$ELK1 O2	160/1176	8.13 e-55
3	NRF1	hsa	RCGCANGCGY V$NRF1 Q6	121/894	1.03 e-40
4	FOX04	hsa	TTGTTT V$FOX04 O1	157/2037	5.28 e-24
5	NFY	hsa	GATTGGY V$NFY Q6 O1	101/1141	3.56 e-19
6	GABP	hsa	MGGAAGTG V$GABP B	76/744	9.21 e-18
7	unknown	hsa	TGCGCANK unknown	63/537	1.2 e-17
8	NFAT	hsa	TGGAAA V$NFAT Q4 O1	133/1871	2.5 e-17
9	LEF1	hsa	CTTTGT V$LEF1 Q2	136/1939	2.64 e-17
10	MYC	hsa	CACGTG V$MYC Q2	86/1015	2.55 e-15
	T3R	hsa	V$T3R Q6	17/248	0.0048
	ERR1	hsa	TGACCTTY V$ERR1 Q2	73/1023	8.99 e-10
	ERR1	hsa	V$ERR1 Q2	20/257	0.0007
	ER	hsa	V$ER Q6 O2	16/251	0.011
	ER	hsa	V$ER Q6 O1	16/264	0.0159
	GR	hsa	V$GR O1	19/202	9.76 e-5
	GR	hsa	V$GR Q6	17/267	0.009
	GRE	hsa	V$GRE C	9/124	0.0255
	LXR	hsa	V$LXR Q3	8/74	0.005
	PPARG	hsa	V$PPARG O1	8/46	0.0003
	PPAR	hsa	V$PPAR DR1 Q2	17/257	0.0066
Down-regulated by fetal Triclosan infusion					
	Transcription Factor	Site		Genes/Total	Adjusted probability
1	SP1	hsa	GGGCGGR V$SP1 Q6	319/2891	1.04e-94
2	MAZ	hsa	GGGAGGRR V$MAZ Q6	212/2250	9.23 e-49
3	MYC	hsa	CACGTG V$MYC Q2	135/1015	1.92 e-46
4	LEF1	hsa	CTTTGT V$LEF1 Q2	185/1939	4.6 e-43
5	E12	hsa	CAGCTG V$E12 Q6	201/2450	2.06 e-37
6	ELK1	hsa	SCGGAAGY V$ELK1 O2	130/1176	3.5e-36
7	FOX04	hsa	TTGTTT V$FOX04 O1	167/2037	1.11 e-30
8	NRF1	hsa	RCGCANGCGY V$NRF1 Q6	103/894	5.75 e-30
9	PAX4	hsa	GGGTGGRR V$PAX4 O3	125/1278	1.17 e-29
10	SREBP1	hsa	TCANNTGAY V$SREBP1 O1	72/466	1.19 e-28
	ERR1	hsa	TGACCTY V$ERR1 Q2	106/1023	4.24 e-27
	ERR1	hsa	V$ERR1 Q2	32/257	9.22 e-11
	ER	hsa	V$ER Q6 O1	29/264	9.70 e-9
	ER	hsa	V$ER Q6	26/273	7.32 e-7
	ER	hsa	V$GR Q6 O2	22/251	1.53 e-5
	GR	hsa	V$GR Q6 O1	22/276	5.88 e-5
	GR	hsa	V$GR Q6	20/267	0.0003
	GR	hsa	V$GR O1	14/202	0.0038
	GRE	hsa	V$GRE C	9/124	0.0138
	PPAR	hsa	V$PPAR DR1 Q2	24/257	2.53 e-6
	PPARA	hsa	V$PPARA O2	10/127	0.0057
	PPARG	hsa	V$PPARG O1	5/46	0.0138
	T3R	hsa	V$T3R Q6	19/248	0.0003
	LXR	hsa	V$LXR DR4 Q3	8/91	0.0069
	LXR	hsa	V$LXR Q3	7/74	0.0076
Up-regulated by maternal Triclosan infusion					
	Transcription Factor	Site		Genes/Total	Adjusted probability
1	SP1	hsa	GGGCGGR V$SP1 Q6	152/2891	2.11 e − 35
2	MAZ	hsa	GGGAGGRR V$MAZ Q6	115/2250	5.15 e-25
3	E12	hsa	CAGGTG V$E12 Q6	105/2450	4.56 e-17
4	NRF1	hsa	RCGCANGCGY V$NRF1 Q6	56/894	1.88 e-15
5	FOX04	hsa	TTGTTT V$FOX04 O1	90/2037	1.88 e-15

(*Continued*)

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Table 2. Continued

		Up-regulated by fetal Triclosan infusion			
6	ELK1	hsa	SCGGAAGY V$ELK1 O2	65/1176	1.88e-15
7	E2F1	hsa	V$ERF1 Q6	29/228	1.88e-15
8	LEF1	hsa	CTTTGT V$LEF1 Q2	86/1939	5.45 e-15
9	E2F1	hsa	V$ERF1 Q3	29/240	5.45 e-15
10	ETS2	hsa	RYTTCCTG V$ETS2 B	58/1074	2.08 e-13
	ERR1	hsa	TGACCTY V$ERR1 Q2	29/1023	0.009
	GR	hsa	V$GR Q6	13/267	0.00 019
	GRE	hsa	V$GRE C	7/124	0.0111
	GR	hsa	V$GR O1	9/202	0.0149
	GR	hsa	V$GR Q6 O1	10/276	0.0308
	PPAR	hsa	V$PPAR DR1 Q2	12/257	0.0040
	PPARA	hsa	V$PPARA O2	7/127	0.0121
	ER			none	
	PPARG			none	
	T3R			none	
	LXR			none	
Down-regulated by maternal Triclosan infusion					
	Transcription Factor	Site		Genes/Total	Adjusted probability
1	SP1	hsa	GGGCGGR V$SP1 Q6	162/2891	1.17 e − 43
2	E12	hsa	CAGGTG V$E12 Q6	117/2450	2.64 e-24
3	ERR1	hsa	TGACCTY V$ERR1 Q2	72/1023	9.61 e-24
4	NFAT	hsa	TGGAAA V$NFAT Q4 O1	95/1871	2.1 e-21
5	MYC	hsa	CACGTG V$MYC Q2	68/1015	2.35 e-21
6	LEF1	hsa	CTTTGT V$LEF1 Q2	94/1939	6.14 e-20
7	unknown	hsa	AACTTT unknown	90/1859	4.81 e-19
8	NFY	hsa	GATTGGY V$NFY Q6 O1	65/1141	6.46 e-17
9	ELK1	hsa	SCGGAAGY V$ELK1 O2	66/1176	6.52e-17
10	AP4	hsa	CAGCTG V$AP4 Q5	75/1502	1.55 e-16
	ERR1	hsa	V$ERR1 Q2	21/257	2.16 e-8
	ER	hsa	V$ER Q6 O1	14/264	0.0004
	ER	hsa	V$ER Q6	13/273	0.0015
	ER	hsa	V$GR Q6 O2	12/251	0.0019
	GR	hsa	V$GR Q6	11/267	0.0078
	GR	hsa	V$GR O1	8/202	0.0249
	GRE	hsa	V$GRE C	6/124	0.023
	T3R	hsa	V$T3R Q6	10/248	0.0121
	PPAR	hsa	V$PPAR DR1 Q2	13/257	0.001
	PPARA	hsa	V$PPARA O2	7/127	0.0084
	LXR	hsa	V$LXR DR4 Q3	10/91	1.38 e-5
	LXR	hsa	V$LXR Q3	7/74	0.0007

Main effects of both maternal and fetal TCS infusions

The GSEA performed with all the genes, and the ontology analysis performed only with the DEG, show that some biological processes are equally affected by both maternal and fetal TCS infusion, while others appear to be affected only if the TCS is infused to the ewe or to the fetus. To explore this concept, we elaborated heat maps with the genes of selected biological processes (displayed in Figure 3). For the main biological processes induced by TCS infusion, the heat maps show that genes related to cell signaling (Figure 3A) and feeding behavior (Figure 3B) are stimulated by TCS infusion in both fetus and mother. Genes involved in inflammatory response (Figure 3C) are induced by fetal TCS infusion rather than by maternal TCS infusion; on the other hand, genes related with the reproductive process (Figure 3D) are induced in maternal TCS infusion. For selected biological processes downregulated by TCS infusion, the heat maps suggest that steroid metabolic process (Figure 3E), lipoprotein metabolic process (Figure 3F) and fatty acid metabolic process (Figure 3G) are inhibited by both maternal and fetal TCS infusion. Glucose metabolic process (Figure 3H) is inhibited by maternal TCS infusion rather than fetal infusion, while chromatin modification (Figure 3I) and central nervous system (CNS) development (Figure 3J) are inhibited by fetal TCS infusion.

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Quantitative PCR confirmation of altered genes related to fatty acid metabolism and appetite

Differential expression of several key regulator genes was confirmed by qRT-PCR. Expression of FASN and ASIP were significantly decreased by either fetal or maternal infusion of TCS (Figure 4 A-B, respectively), while expression of POMC and AGRP were significantly increased by either maternal or fetal infusion of TCS (Figure 4C and D).

Discussion

The results of this study reveal that two days of low dose TCS infusion – designed to mimic exposure from the use of personal care products containing TCS – induces strong changes in gene expression in the fetal hypothalamus, effects that are even more profound after direct fetal exposure. Conceptually, the changes in hypothalamic gene expression may reflect the neuroendocrine response to the disturbances in fetal physiology after exposure to TCS.

TCS is a known inhibitor of the enoyl reductase activity of the Type II Fatty Acid Synthase enzyme compled in bacteria: it is this action as enzyme inhibitor that is the basis of its antibacterial action (3). TCS also inhibits the Type I Fatty Acid Synthase in mammals (encoded by the FASN gene), although at much higher concentrations (27). Interestingly, the major transcriptomics responses to both maternal and fetal TCS infusion are related to fatty acid

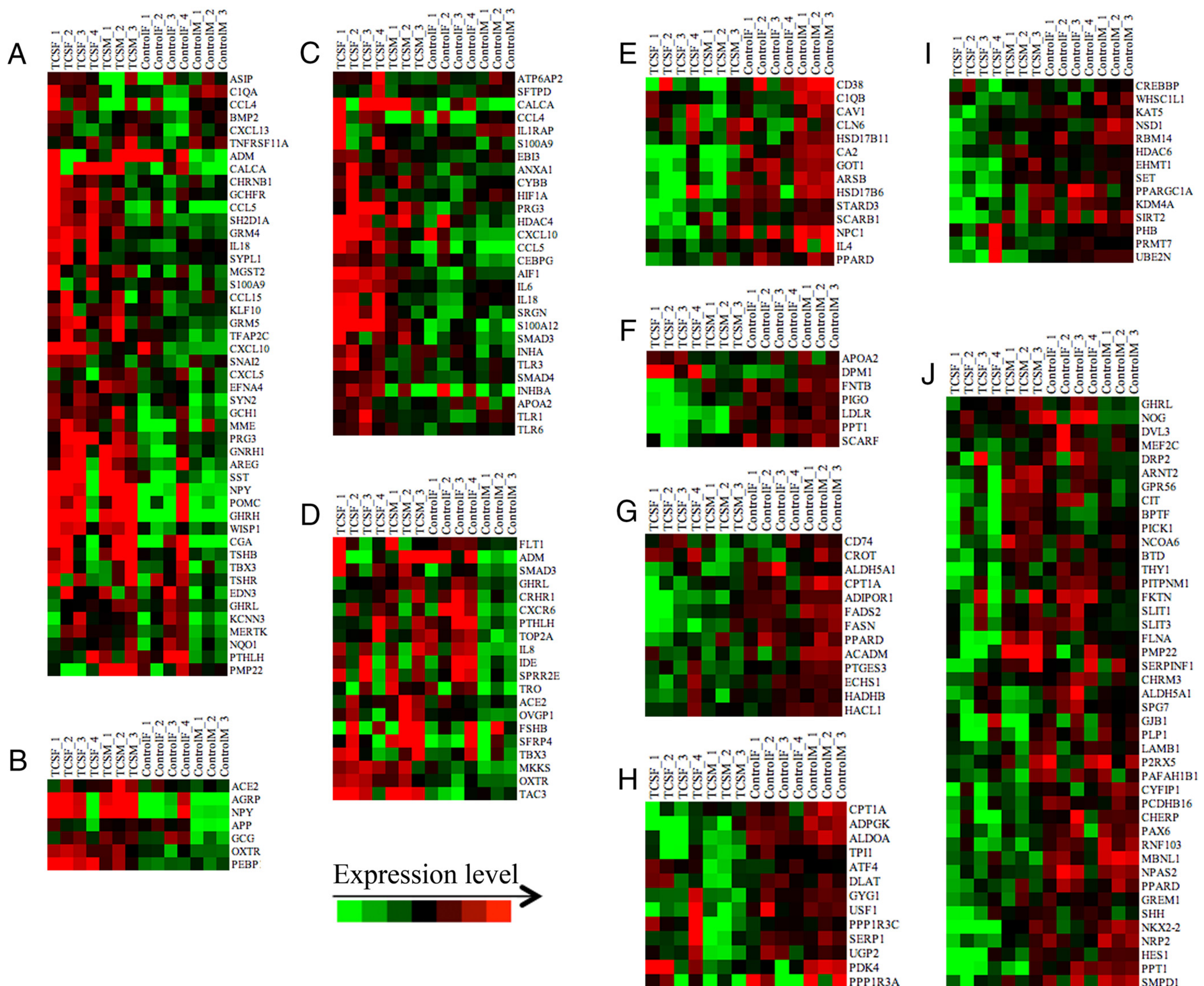

Figure 3. Heat maps of core genes involved in selected Biological Processes (BP) affected by maternal or fetal Triclosan (TCS) infusion. Heat maps from A to D represent BP increased by TCS infusion while heat maps from E to J belong to BP decreased by TCS. These BP are: A) Cell signaling; B) Feeding behavior; C) Inflammatory response; D) Reproduction; E) Steroid metabolic process; F) Lipoprotein metabolic process; G) Fatty acid metabolic process; H) Glucose Metabolic Process; I) Chromatin Modification and J) Central Nervous System Development.

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and lipoprotein metabolism and regulation of food intake and energy balance. TCS exposure decreased the expression of genes related to the metabolism of lipoproteins (Figure 3D), fatty acids (Figure 3G) and glucose (Figure 3H), including a reduction in genes associated with fatty acid metabolism, including a reduction of fatty acid synthase (FASN), as determined by the array and confirmed by qRT-PCR (Figure 4A). While we do not know if the effect of TCS on fetal hypothalamic FASN is direct, several of the genes affected by TCS are known to be related to each other mechanistically. FASN gene transcription is increased by an additive effect of insulin and agouti protein, the product of the ASIP gene, since FASN promoter has an agouti response region distinct from the insulin-response element (28); ASIP was downregulated by (maternal or fetal) infusion of TCS (Figure 4B).

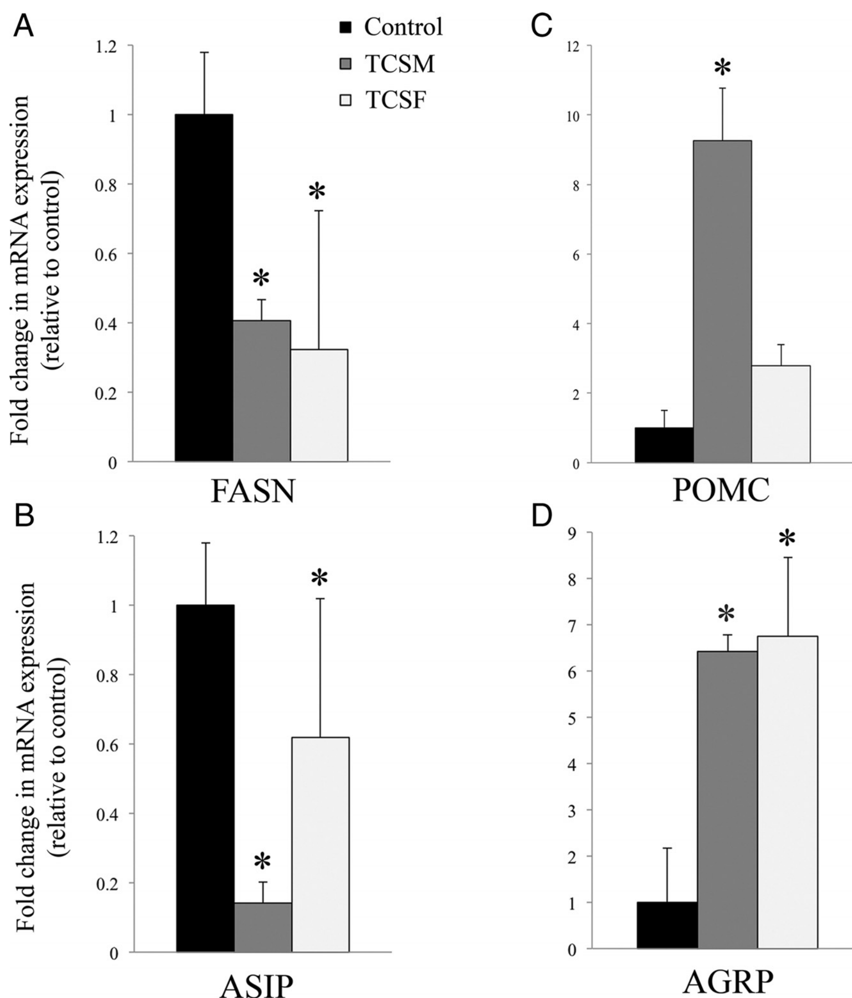

Figure 4. mRNA expression measured by qRT-PCR in fetal hypothalami after maternal (TCSM) or fetal (TCSF) Triclosan infusion. mRNA expression for each group is shown as fold change with respect to the controls for the downregulated genes: A) fatty acid synthase (FASN) and B) agouti signaling protein (ASIP); and up-regulated genes: C) proopiomelanocortin (POMC) and D) agouti-related protein (AGRP). *Statistically significant difference ($P < .05$) in mRNA expression between TCS and control group

TCS also altered the expression of genes important for regulation of food intake in postnatal animals. In the present study we found that both AGRP mRNA and POMC abundance were significantly increased (Figure 4C and D). The agouti-related protein, encoded by the AGRP gene, is known to be an important neuropeptide with respect to hypothalamic control systems (29). AGRP is expressed by the arcuate neurons (30) that coexpress NPY. These neurons are found in the arcuate nucleus as an adjacent but distinct population from those that produce POMC, the precursor of the melanocortin αMSH (31). Both ASIP and AGRP are inverse agonists at the melanocortin receptor, MC4R in vitro (32). The neuropeptides NPY and AGRP increase feed intake (orexigenic), whereas α-MSH (cleaved from POMC) is anorectic, (reviewed in (33)). Although AGRP and POMC are expressed in discrete populations of neurons in the adult, and have opposing effects on food consumption, in the developing brain these neurons develop from the same lineage. The development of hypothalamic neurons is not completely understood, but in rodents neurogenin 3 (NEUROG3) is known to be expressed in the progenitor neurons, and appears to lead to greater POMC expression and reduced NPY and AGRP expression (34), although there are some POMC neurons that do not arise from neurogenin positive neurons. Neurogenin is on our array and was not differentially expressed with TCS exposure, consistent with its expression in the period of development preceding neuropeptide expression. In rodents, maturation of connections of mature neurons expressing NPY/AGRP or POMC occurs postnatally (35), but these components of the neuronal network that regulate appetite are expressed in the arcuate nucleus of the ovine fetal hypothalamus from at least 110 days of gestation (36). Expression of the orexigenic genes NPY and AGRP in the arcuate of the late gestation fetal sheep are increased by maternal undernutrition (37), whereas glucose infusion increased fetal POMC, but not NPY or AGRP expression (38). Increased expression of mRNA for both the orexigenic and anorexic

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neuropeptides after TCS infusion suggests that neuronal maturation in the ovine fetal hypothalamus may be altered. Expression of other hypothalamic neurohormones or neuropetides, were also increased by maternal TCS infusion, including GHRH, GNRH, IGF2, prolactin releasing hormone (PRLH) and somatostatin (SST), suggesting that multiple neuronal populations are affected by TCS exposure. As expression of genes associated with mitosis, cell cycle, and developmental maturation were up-regulated, this suggests that TCS may stimulate expansion of, and neuropeptide expression in a variety of hypothalamic neurons important for growth, energy and food intake postnatally.

Maternal TCS administration increased expression of ghrelin (GHRL) and glucagon-like peptide receptor (GLP1R), which would be expected to have opposing effects on activity of neurons in the orexigenic-anoretic pathways. In postnatal animals ghrelin increases activity of NPY neurons, resulting in increased food intake (reviewed in (39)), whereas GLP-1 inhibits these responses in fasting rats (40). The expression of the neuropeptides is also influenced by leptin and insulin. In the postnatal animal, insulin inhibits the expression of NPY/AGRP neurons and increases the expression of the POMC neurons, whereas leptin has the opposite effect (reviewed in (41)). In keeping with the observation that there was not differential expression of POMC and AGRP or NPY in these fetuses, expression of insulin and leptin signaling molecules did not appear to be differentially expressed after either maternal or fetal infusion of TCS; expression of INSR and IRS1, the insulin receptor and insulin substrate 1, and of LEP and LEPR, leptin and its receptor, were not significantly changed, nor were expression of SOCS3, STAT3 or FOXO1, downstream signaling molecules (40). The postnatal implications of the increases in expression of both sides of this balance, that is, both anorexigenic and orexigenic neuropeptides, and the neuropeptides that stimulate their release in fetal life are not clear. The adjusted array data suggests that the AGRP expression is more markedly increased (8.3 fold) as compared to POMC expression (2 fold) suggesting that the stimulation of orexigenic pathways may be greater. It is unknown however, to what extent maturation of these pathways may be disrupted by TCS, altering the neonates ability to match energy needs and food intake in postnatal life.

Transcription factor analysis revealed a substantial overlap between groups of up- and down-regulated genes. This result may reflect an indirect action of TCS on the fetal hypothalamus, perhaps with changes in gene expression following TCS-mediated alterations in maternal-fetal metabolism. One insight into the action of TCS might be the involvement of SREBP (sterol regulatory element-binding protein, which may reflect changes in local sterol biosynthesis), and GABP (GA binding protein, which may reflect changes in mitochondrial function) in the down- and up-regulation of genes after fetal TCS infusion.

A well-known action of TCS postnatally is disruption of the hypothalamus-pituitary-thyroid hormone axis. Exposure to TCS, either during pregnancy or lactation, has been related to maternal and neonatal hypothyroidism (7–9). These studies established the association of the resulting hypothyroxinemia to increased hepatic catabolism. However, a study by Paul et al (8) suggests that other modes of actions could contribute to the observed hypothyroxinemia. TCS inhibits SULT1E1 (*supra vita*) and SULT1B1, enzymes that catalyzes the sulfonation of iodothyronines -such as the prohormone T4 and the active hormone T3- for their inactivation and excretion (42). In the present study, we found an important downregulation of the hypothalamic thyrotropin-releasing hormone (TRH) receptor (TRHR) when TCS was administered to the pregnant ewe (more than 2 fold, measured by microarray). TRH receptors are located in the lateral hypothalamus and in the ventromedial hypothalamus, areas that are important in food intake and energy balance. Central TRH administration is known to decrease food intake, although the site and mechanism of this action is not well characterized (43). TRH neurons are located in the paraventricular nucleus (PVN) but they could project to the lateral hypothalamus to inhibit the secretion of the orexigenic peptides such as melanin-concentrating hormone (44). NPY neurons in the arcuate nucleus innervate and inhibit TRH neurons in the PVN (45). NPY also reduces pro-TRH processing (46). Although a direct link has not been established so far, an increase of the orexigenic NPY may downregulate TRH receptors. Accordingly, food restriction in rats reduced hypothalamic mRNA levels of both TRHR alpha and beta and TRH, but increased the levels of NPY (47). Thus, the strong downregulation of TRHR in hypothalamus after TCS treatment supports the concept that TCS interrupts fetal energy balance.

Thyroid hormone signaling is essential for brain development since thyroid hormone acts on regulatory genes that shape the brain during fetal life (48). This could explain inhibition of genes related to CNS development after fetal TCS infusion (Figure 3J). While it is interesting that genes containing transcription factor binding site for thyroid hormone receptor were found in all four experimental groups (Table 2), it is not possible to know whether there is a cause-and-effect relationship between tissue T3 concentrations and transcriptomics response without direct measurements of T3 concentrations.

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Conclusion

This study demonstrated that a short-term infusion of TCS, either in the pregnant ewe or in the ovine fetus, induces vigorous changes in the fetal hypothalamic transcriptomics, which are primarily related to pathways important for lipid metabolism, food intake, and energy balance postnatally. While we are mindful of the fact that these are short-term infusions, the pattern of gene expression suggests to us the possibility that exposure to TCS during fetal life might predispose the newborn lamb to disturbances of the food intake and energy homeostasis, the thyroid hormone system and the reproductive function, with possible consequences in the pattern of growth.

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