ORIGINAL ARTICLE

Gestational diabetes exacerbates maternal immune activation effects in the developing brain

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Maternal inflammation and diabetes increase the risk for psychiatric disorders in offspring. We hypothesized that these co-occurring risk factors may potentiate each other. To test this, we maternally exposed developing mice *in utero* to gestational diabetes mellitus (GDM) and/or maternal immune activation (MIA). Fetal mouse brains were exposed to either vehicle, GDM, MIA or GDM+MIA. At gestational day (GD) 12.5, GDM produced a hyperglycemic, hyperleptinemic maternal state, whereas MIA produced significant increases in proinflammatory cytokines and chemokines. Each condition alone resulted in an altered, inflammatory and neurodevelopmental transcriptome profile. In addition, GDM+MIA heightened the maternal inflammatory state and gave rise to a new, specific transcriptional response. This exacerbated response was associated with pathways implicated in psychiatric disorders, including dopamine neuron differentiation and innate immune response. Based on these data, we hypothesize that children born to GDM mothers and exposed to midgestation infections have an increased vulnerability to psychiatric disorder later in life, and this should be tested in follow-up epidemiological studies.

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INTRODUCTION

It is increasingly recognized that the prenatal environment influences the risk for developing disease later in life.¹ Multiple studies have defined links between diverse stressors during pregnancy and the risk to offspring, including neurocognitive problems.^{2,3} The majority of studies linking prenatal stress to developmental origins of disease in offspring have focused on a single type of stress (for example, diabetes or infection). However, human populations are routinely subjected to more than one potential developmental stressor at a time, and recent studies suggest that such interactions are important in the context of future health outcomes (reviewed in Goldstein *et al.*⁴).

Increased risk for psychiatric disease is a result of genetic liability interacting with a variety of environmental perturbations. Numerous gene × environment interactions have been identified, such as *BDNF* variants co-occurring with serious obstetric complications increasing risk for schizophrenia.² Yet, environment × environment interactions on the developing fetus are greatly understudied in comparison. Two commonly co-occurring psychiatric disorder risk factors that alter the *in utero* environment are maternal immune activation (MIA) and gestational diabetes mellitus (GDM).^{5–8}

Both animal and epidemiologic studies support that midgestation influenza infection increases risk for autism and schizophrenia.^{5,9,10} However, unlike some viruses (for example, Rubella, Zika), influenza virus does not cross the placenta, suggesting that the maternal immune response (and not the pathogen) is the key to pathology in this setting.^{11,12} This process

of viral-induced MIA is modeled in pregnant mice using viral mimetics such as polyinosinic:polycytidylic acid (poly(I:C)), a synthetic, double-stranded RNA agonist of Toll-like receptor 3.^{11,12} In fact, it has been repeatedly established with multiple models that MIA disrupts normal fetal brain development^{10,13} and that mice born to mothers exposed to MIA in adulthood display behavioral abnormalities that are relevant to both schizophrenia and autism spectrum disorder.^{12,14}

GDM is the most common metabolic disorder during pregnancy, causing considerable morbidity and long-term complications for both mother and child.¹⁵ Recent birth cohort studies have demonstrated that diabetes during pregnancy increases risk for autism spectrum disorder and potentially schizophrenia.^{8,16,17} and offspring of GDM rodent models exhibit a wide-range of neurodevelopmental, behavioral and cognitive abnormalities.^{18,19} Furthermore, hyperglycemia and hyperinsulinemia can increase systemic inflammation and exaggerate and/or prolong responsiveness to proinflammatory stimuli, supporting a possible interaction of GDM and MIA.^{20–24}

Disruptions to the normal immune homeostasis within the fetus or placenta can disrupt neurodevelopment and predispose to psychiatric disorders.^{7,25} This inflammation does not have to be chronic or excessive to predispose to psychiatric disorders but depends greatly on the contextual factors it is combined with.^{26,27} Both MIA and GDM have their peak influence at midgestation,^{8,28} and previous studies suggest that GDM may interact with the proinflammatory state produced by MIA.^{20–23,29,30} Resulting changes in the maternal state can potentially disrupt the *in utero*

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environment, such as increases in interleukin (IL)-6, that can alter placental function and development and, in turn, might negatively affect fetal brain development.^{31–33} The purpose of this study was to interrogate the interaction between MIA and GDM during prenatal development, hypothesizing that these two common and potentially co-occurring factors may potentiate each other.

MATERIALS AND METHODS

Animal procedures

All animal procedures were approved by the Vanderbilt Animal Care and Use Committee. Mice were housed in ventilated cages under standard laboratory conditions and allowed *ad libitum* access to food and water. Female and male C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) for timed pregnant breeding. Control-fed female mice received standard chow throughout the experiment (5LOD, Lab Diet, St Louis, MO, USA). To induce GDM, females received a 60% calories-by-fat diet (58Y1, Test Diet, St Louis, MO, USA) from 4 weeks of age until 10 weeks of age, continuing the high-fat diet throughout pregnancy. At 10 weeks of age, mice were mated overnight, and the presence of a vaginal plug marked gestational day 0.5 (GD0.5). Pregnant females were left undisturbed except for cage changes at GD9.5 and weight measurements.

GDM and control females were assigned to either saline or poly(I:C) treatment groups. Samples were balanced between MIA and control each week, providing randomization within the constraints of the breeding scheme. This design created four groups: control-fed saline (SAL or Control), high-fat-fed saline (GDM), control-fed poly(I:C) (MIA), and high-fat-fed poly(I:C) (GDM+MIA). GD12.5 was chosen as the midgestation timepoint in mice that is neurodevelopmentally distinct from midgestation in humans. At GD12.5, pregnant females were injected intraperitoneally with either sterile saline or 20 mg kg⁻¹ poly(I:C) potassium salt (Sigma Aldrich, St Louis, MO, USA) in sterile saline that was based on the weight of the poly(I:C) itself. Pregnant mice were killed 3 h after injection at GD12.5. Data from a second cohort of pregnant mice killed 4 days after injection at GD16.5 were used to evaluate the evolution of these effects after the immune stimulus passed. Sex genotyping was performed for each embryo using a previously published protocol.³⁴ Each group at each time point (4 groups, 2 time points) contained 9 pregnant females with the exception of GD12.5 MIA that only contained 8 pregnant females, making a total of 71 females. Sample size was chosen based on empirical data from previous literature findings, as well as power calculations.

Glucose tolerance testing and body composition analysis

A separate cohort of female mice underwent the same feeding, mating and treatment regime as described above for the purposes of confirming the presence of GDM via metabolic testing and glucose tolerance testing ($n=6$ per group). At GD12.5, pregnant control-fed and high-fat-fed females were fasted for 6 h (0700–1300 h). Body composition was then measured via whole-body nuclear magnetic resonance (Minispec Model mq7.5, Bruker Instruments, Billerica, MA, USA) with the assistance of the Vanderbilt Mouse Metabolic Phenotyping Center (supported by U24 DK059637). At 1300 h, fasting glucose and insulin samples were taken followed by intraperitoneal glucose tolerance testing with 2 g dextrose per kg body weight that was performed according to standardized procedures.³⁵ To evaluate the effects of time on high-fat diet in the absence of pregnancy, one 10–12-week-old nonpregnant control group ($n=8$) was utilized as the control for nonpregnant high-fat-fed females aged 10 weeks ($n=7$) and 12 weeks ($n=8$).

Antibody-conjugated bead array immune profiling

Blood was collected via cardiac puncture and allowed to clot at room temperature for 30 min. Samples were centrifuged at 8000 r.p.m. for 10 min at 4 °C. Supernatants were centrifuged again at 8000 r.p.m. at 4 °C for 10 min to remove residual red blood cells. Serum was stored at -80 °C until antibody-conjugated bead array analysis. Luminex (EMD Millipore, Darmstadt, Germany) analysis was performed by the Vanderbilt Hormone Core (funded by DK059637 and DK020593). The Luminex multiplex panels (EMD Millipore) used for analysis of the maternal serum were the mouse metabolic hormone panel (insulin and leptin: Cat. No. MMHMG-44K), mouse adiponectin (Cat. No. MADPNMG-70K-01) and mouse cytokine/chemokine panel 14 plex (eotaxin, IFN- γ , IL-1 α , IL-1 β , IL-4, IL-6, IL-10, IL-12p40, IL-13, IL-17, KC, MCP1, RANTES and TNF α : Cat. No. MCYTOMAG-70K).

When values fell outside the normal range of the assay (that occurred for some serum samples for monocyte chemoattractant protein-1 (MCP1) and IL-6), they were analyzed as the highest detectable or lowest detectable value as appropriate. As MCP1 levels were the most consistently upregulated, 3 poly(I:C)-treated dams killed at 3 h after exposure below 2 s.d. from the MCP1 mean were excluded from the study as nonresponders or poor injections.

RNA isolation and gene expression analysis

The right hemisphere from each flash frozen embryonic brain (telencephalon and diencephalon) was utilized for RNA isolation. RNA was isolated in 1 ml Trizol, following the protocol given by the manufacturer (Invitrogen, Carlsbad, CA, USA). Samples then underwent a Qiagen RNeasy clean-up, following the manufacturer's instructions (Qiagen, Hilden, Germany). RNA concentration was measured with a Thermo Scientific Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). Equal amounts of RNA (1250 ng) from each embryo were pooled for a litter sample. These pooled samples again underwent a Qiagen RNeasy clean-up to ensure absence of contaminants. Nanodrop was utilized to measure the final concentration and ensure purity (260/280 values above 2 and 260/230 values above 1.5). Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) was utilized to ensure RNA integrity, with all RNA integrity numbers between 9.5 and 10.

From each pooled litter, 150 ng of purified RNA was used for unblinded Nanostring nCounter gene expression analysis (<http://www.nanostring.com/applications/technology>). Two panels (mouse inflammation V2 panel—254 genes—and a custom inflammation and neurodevelopment panel—46 genes; Supplementary Table 1) were utilized to determine gene expression that was performed according to the manufacturer's instructions. Target genes were prioritized based upon three criteria: (1) importance in innate and adaptive immune responses to infection, (2) importance in neural development and (3) existing knowledge regarding changes in gene expression reported in previous studies of MIA or GDM alone. The raw 'count'—the number of times the reporter probe was detected in each sample—was normalized to positive and negative spike-ins as well as 5 housekeeping genes (*Cltc*, *Gapdh*, *Gusb*, *Hprt* and *Tubb5*). After normalization, the sum of all counts was calculated and counts $> \log_2(3.5)$ were further used for comparison.

Statistical approaches

Power analyses were performed using the NCSS sample size software PASS (www.ncss.com/software/pass/). We had $>90\%$ power to detect one unit difference in outcome with an estimated s.d. of 0.2 with a false discovery rate of 0.05. The power of study further improved because of pooling RNA from each litter, thus reducing the effects of individual variability.

To investigate the effects of each exposure, a one-way analysis of variance with Bonferroni *post hoc* analysis was used for the measured fetal or maternal parameters, with $P < 0.05$ considered significant. For transcriptional analysis, unpaired Student's *t*-test was utilized to compare values between control and each condition, and significant genes ($P < 0.05$ and false discovery rate analysis < 0.01) were used for differential expression analysis. Comparisons between multiple conditions utilized two-way analysis of variance with Tukey's multiple comparison correction with significance of $P < 0.05$. Statistics analysis was performed using Graphpad Prism (La Jolla, CA, USA). Gene ontology analysis and weighted functional gene network mapping was performed for each condition with genes at least 1.15-fold change compared with control mice using DAVID Bioinformatics resources and Cytoscape Genemania application, respectively.^{36–39} Venn diagram analyses were generated using Venny (<http://bioinfogp.cnb.csic.es/tools/venny/>). Data represent average of eight or nine biological replicates.

RESULTS

High-fat diet and poly(I:C) treatment affect dam weight

Poly(I:C) treatment has been shown to cause a transient increase in cytokine production, accompanied by anorexia, fever and sickness behaviors that usually resolves in 24–48 h. The poly(I:C)-treated dams in our study either lost weight or failed to gain weight compared with saline-treated dams (weight change $\pm$ s.e.m.: SAL = 0.60 ± 0.33 , MIA = -0.83 ± 0.34 ; GDM = 1.33 ± 0.25 ; GDM + MIA = -0.67 ± 0.31 ; $P < 0.05$). Neither diet nor treatment altered the average number of embryos per litter or the male/female ratio (Supplementary Table 2).

Figure 1. High-fat diet produces a diabetic phenotype midgestation. Body composition, 6 h fasting glucose and insulin and glucose tolerance measurements were performed on a cohort of high-fat (HF)- and control (CTR)-fed gestational day 12.5 (GD12.5) pregnant dams ($n=6$ per group, HF and CTR in graph, respectively). **(a)** HF-fed dams showed significant increases in body weight at GD9.5 and GD12.5. **(b)** Whole-body nuclear magnetic resonance (NMR) at GD12.5 showed significant changes in % body mass in HF dams with increased % fat mass and decreased % lean body mass. **(c, d)** The 6 h fasting blood glucose and serum insulin were both significantly increased in HF fat dams. **(e–g)** After a 6 h fast, dams were injected intraperitoneally with 2 g dextrose per kg body weight. HF-fed dams demonstrated a higher peak and prolonged increase in blood glucose with significant differences from control dams observable at 0, 10, 20, 30, 60 and 75 min after injection, supported by a significantly increased area under the curve. A significant heightened insulin response that was maintained up until the 2 h end point was also observed in HF dams. Error bars represent s.e.m. Significance determined by unpaired Student's *t*-test with Welch's correction in all assays (* $P < 0.05$, $n=6$ dams per group).

High-fat diet leads to GDM midgestation

Although the dams were on a 60% calories-by-fat diet for 6 weeks before mating and throughout pregnancy, these mice were not obese compared with the controls. Compared with control-diet-fed dams, the high-fat-fed dams had increased body weight (weight $\pm$ s.e.m. (g): control = 25 ± 0.8 , high fat = 29.3 ± 0.9 ; $P < 0.01$) and percent fat mass (% $\pm$ s.e.m.: control = 10.3 ± 1.1 ,

high fat = 19.2 ± 0.9 ; $P < 0.0001$) at GD12.5 (Figures 1a and b). Even so, this model successfully produced a GDM phenotype. To demonstrate this, glucose tolerance testing was conducted on mice fed normal- or high-fat diets. The dams fed high-fat diet had both impaired glucose (glucose $\pm$ s.e.m. (mg dl^{-1}): control = 141 ± 6 , high fat = 197 ± 9 ; $P < 0.0001$) and increased insulin (insulin $\pm$ s.e.m.: control = 0.44 ± 0.1 , high fat = 0.78 ± 0.1 ; $P < 0.01$) levels after 6 h of

Figure 2. Maternal immune activation (MIA) and gestational diabetes mellitus (GDM) together produce metabolic and inflammatory disruption of maternal serum factors. Maternal serum collected 3 h after injection on gestational day 12.5 (GD12.5) was analyzed for chemokine, cytokine and adipokine levels via antibody-conjugated bead assay. (a–i) Significant increases were observed with MIA and MIA+GDM in most cytokines/chemokines measured without any difference between MIA and MIA+GDM or attributable to GDM alone. Only (e) interleukin-17 (IL-17) was significantly increased by GDM alone. (j) GDM alone did not alter nonfasting serum insulin levels, but GDM+MIA produced a significant increase. (k) The proinflammatory satiety hormone leptin was significantly increased by GDM, and this increase was even more prominent in the GDM+MIA group. Error bars represent s.e.m. Significance determined by one-way analysis of variance (ANOVA) with Bonferroni *post hoc* analysis in all assays. Serum was collected from dams whose embryos were utilized for gene expression analysis (* $P < 0.05$, SAL $n = 9$, MIA $n = 8$, GDM $n = 9$, GDM+MIA $n = 9$).

fast (Figures 1c and d). When challenged with 2 g kg⁻¹ dextrose after the 6 h fast, dams fed high-fat diet displayed significant glucose intolerance (area under the curve $\pm$ s.e.m.: control = 32 653 $\pm$ 2361, high fat = 46 528 $\pm$ 3172; $P < 0.01$) and exaggerated glucose-induced insulin secretion that remained high even at 2 h after injection (insulin $\pm$ s.e.m. (ng ml⁻¹): control = 0.42 $\pm$ 0.1, high fat = 1.54 $\pm$ 0.3; $P < 0.01$; Figures 1e–g). Confirming that this phenotype is truly gestation dependent, neither high-fat- nor control-diet-fed dams demonstrated this phenotype at GD0.5 (Supplementary Figure 1). Similarly, age-matched, nonpregnant high-fat- and control-diet-fed dams at GD12.5 did not report differences in these GD measures (Supplementary Figure 2).

MIA produces an acute increase in maternal cytokines and chemokines

To assess the poly(I:C)-induced immune response in pregnant dams as well as differences between normal and GDM mice, cytokines and chemokines were measured in maternal serum at

the time of killing. Similar to previous observations of the acute response to poly(I:C)^{40,41} 3 h after poly(I:C) exposure, MIA and GDM+MIA dams demonstrated increases in eotaxin, IL-1 β , IL-6, IL-10, IL-12p40, IL-17, KC, TNF- α , MCP1 (CCL2) and RANTES (CCL5) ($P < 0.05$; Figure 2). No significant differences were found with interferon- γ (IFN γ), IL1- α , IL1- β or IL-4 (Supplementary Table 3). Perhaps not surprisingly, by GD16.5 almost no serum factor changes with MIA were observed, suggesting that the effects of single poly(I:C) injection had largely subsided by 4 days after exposure (Supplementary Figure 3).

Maternal metabolic hormones are altered by GDM+MIA

Nonfasting serum insulin levels showed only a nonsignificant increase in the GDM dams, whereas the combination of GDM and MIA exposure produced a strong, significant increase in serum insulin levels (insulin $\pm$ s.e.m. (pg ml⁻¹): SAL = 523 $\pm$ 102, MIA = 575 $\pm$ 135; GDM = 800 $\pm$ 94, GDM+MIA = 1353 $\pm$ 203; $P < 0.05$; Figure 2j). Interestingly, the immune-activating, satiety-inducing hormone leptin

was significantly increased by GDM and further increased by combination of GDM and MIA exposure, but was not altered by MIA alone (leptin $\pm$ s.e.m. (pg ml⁻¹): SAL = 1358 $\pm$ 94, MIA = 1389 $\pm$ 256; GDM = 5064 $\pm$ 977, GDM+MIA = 8316 $\pm$ 1173; P < 0.05; Figure 2k). This increase persisted in the GDM+MIA group at GD16.5 (leptin $\pm$ s.e.m. (pg ml⁻¹): SAL = 1320 $\pm$ 203, MIA = 1184 $\pm$ 188; GDM = 3288 $\pm$ 569, GDM+MIA = 3940 $\pm$ 1177, P < 0.05; Supplementary Figure 3). In contrast, MIA exposure alone did not significantly alter leptin or insulin levels at either GD12.5 or GD16.5.

Inflammation and development-related gene expression in the embryonic brain is disrupted by GDM and MIA

Our gene expression investigation focused on a panel of literature- and hypothesis-derived transcripts that encompassed genes involved in inflammation, neurodevelopment and neuronal glucose transporters. Both high-fat-diet-induced GDM and MIA resulted in altered gene expression in the fetal brain at GD12.5. When comparing significantly different (P < 0.05, false discovery rate < 0.01) gene expression changes between SAL, MIA, GDM or GDM+MIA in an unsupervised hierarchical clustering analysis, each of the four treatment groups demonstrated specific gene expression profiles (Supplementary Figure 4 and Supplementary Table 4).

GDM alone displayed significant differences in gene expression compared with SAL. Neurodevelopmental genes involved in patterning (*En1*, *En2*) and cholinergic signaling (*Chat*) were repressed.⁴² A subset of inflammation-associated genes were induced (for example, *Cls*, *Ccl8*, *Ifit3*, *Mx2*), whereas others were repressed (for example, *Ccl21a*, *Ifi44*, *Tgfb2*, *Tgfb3*), with these genes functioning in IFN/antiviral response, growth, cell cycle regulation and apoptosis.⁴³ Moreover, GDM increased *Vegfa* expression that is associated with a hypoxic environment (Figure 3a).⁴⁴

Our lab has previously characterized MIA-triggered gene expression changes in the fetus after maternal poly(I:C) treatment at GD12.5.¹⁰ Here again, we report altered inflammation-responsive gene expression with MIA alone compared with SAL (Figure 3b). We noted both induction of antiviral/IFN response genes (for example, *Ifi44*, *Ifit3*, *Mx2*, *Oasl1*) and *Alox12* that produces a lipid peroxidation enzyme.^{43,45,46} Repressed inflammation genes observed participate in cell cycle regulation/apoptosis (*Bcl6*, *Cd55*, *Map3k5*, *Mef2b*, *Myc*), cell growth (*Pdgfra*, *Tgfb3*) and intracellular signaling pathways (*Creb1*, *Fxyd2*).^{47–49} Yet, the MIA- and GDM-dependent expression profiles were also distinct, as MIA repressed many unique neurodevelopment-associated transcripts (for example, *Bdnf*, *Dckl1*, *Gabra2*, *Ncam1*, *Syt1*).⁵⁰ Furthermore, *Vegfa* was induced by MIA, suggesting alteration in oxygen and nutrients reaching the fetal brain.^{44,51}

GDM potentiates MIA-induced gene expression changes

At 3 h following injection, poly(I:C) treatment in the context of GDM produced marked induction of many inflammation and neurodevelopment genes (Figure 3c). GDM+MIA both induced (*Foxg1*, *Gad1*, *Vamp1*) and repressed (*En1*, *En2*, *Fgf8*, *Pax5*) subsets of neurodevelopment genes involved in patterning and migration.^{50,52–54} Moreover, both the constitutively expressed GLUT1 and high-affinity neuronal-specific GLUT3 genes (*Slc2a1*, *Slc2a3*) and hypoxia-associated *Vegfa* were induced in GDM+MIA. Inflammation genes induced by GDM+MIA are mostly involved in antiviral/IFN response (for example, *Ifi44*, *Ifit3*, *Ifitm3*, *Mx2*, *Oasl1*) and the generalized innate immune response (for example, *Ager*, *Ccl2*, *Ccl8*).^{43,45,49} Contrastingly, several apoptosis/cell cycle regulation-associated genes (for example, *Mef2a*, *Mef2b*, *Tradd*) were repressed by GDM+MIA.^{43,47,48} GDM+MIA also repressed genes critical for intracellular signaling pathways associated with pro- and anti-inflammatory processes (*Map3k5*, *Nfe2l2*), immune response (*Cd55*, *Itgb2*) and neurodevelopment

(*En1*, *En2*, *Fgf8*, *Pax5*).⁵⁵ Altogether, our data suggest further disruption of the fetal brain by the complex metabolic, inflammatory derangement produced by GDM+MIA, including both unique and differentially altered MIA- and/or GDM-associated transcription changes (Figure 4). This unique phenotype is enriched in dopamine neuron differentiation and multiple inflammatory pathways (Figure 5).

At GD16.5, smaller and fewer changes in relative gene expression were detected with MIA and GDM+MIA that should be expected as the acute immune response induced by poly(I:C) exposure passes after 24–48 h (Supplementary Figure 5 and Supplementary Table 5). In contrast, we observed a similar number of gene expression changes produced by GDM alone at GD16.5 compared with those observed at GD12.5, including induction of neurodevelopment genes (*En2*, *Pax5*) and both repression (*Ccr1*, *Cd163*, *Itgb2*) and induction (*Hras1*, *Ptger3*) of inflammation genes. This supports continued effects of GDM on the fetal brain. This evolving phenotype reflected ongoing changes in neurodevelopment and immune response in the developing brain.

DISCUSSION

There is abundant epidemiological evidence supporting an association between either GDM or infection-associated MIA and adverse neurocognitive outcomes in offspring, including autism and schizophrenia.^{5,8,9,16} However, the extent to which these two stressors interact has not been closely evaluated to date. Potential interactions between GDM and MIA are of great clinical relevance in developed countries such as the United States, where the incidence of GDM is increasing,¹⁵ and where infections pose a persistent threat to pregnant mothers. In low- or middle-income countries this may be even more important in light of high rates of infectious diseases and recent transitions from undernutrition to excess adiposity.⁴

Herein we present new evidence for a significant interaction between GDM and MIA in the developing fetal brain. These two environmental insults commonly occur during gestation and are associated with increased risk for psychiatric disorders later in life.^{3,5,8,9,56} The combination of these insults demonstrate that (1) GDM and MIA individually alter circulating factors in maternal serum and alter inflammation-associated and neurodevelopmental embryonic gene expression, (2) GDM and MIA have a complex maternal interaction that produces a novel transcriptional phenotype and (3) these novel transcriptional changes are associated with pathways implicated in psychiatric disorders like dopamine neuron differentiation and innate immune response. These findings provide insight into prenatal interactions that may increase risk for psychiatric disorder development later in life.

Our maternal serum data confirm that MIA induces a proinflammatory state. We do not find this altered by co-occurrence with GDM. Yet, results from fetal brain gene expression suggest that metabolic stress (for example, GDM) primes the fetus for an altered response to MIA. GDM has been associated with low-grade inflammation²⁹ that might have the potential to produce a 'primed' state, altering and potentially exacerbating the response to additional inflammatory stimuli.^{25,29} In fact, multiple environmental stressors such as midgestation maternal infection, perinatal hypoxia or early childhood trauma are often considered cofactors or triggers for this 'primed' state that may have arisen from genetic and/or environmental insults.^{57,58}

In addition, these studies confirmed previous findings in pregnant mice exposed to MIA, where similar transient increases in cytokine and chemokine levels were observed during the acute response to poly(I:C).^{40,41,59} In contrast to the acute phase, 1–8 days after injection most studies have reported subtle or no increases in cytokines or chemokines in maternal serum. This is

gene expression changes in the brains of offspring, we believe that the data argue that this proinflammatory cytokine environment poses a significant risk for adverse health outcomes in later life that should be investigated with additional Toll-like receptor agonists in future studies.^{14,61}

Figure 4. Gestational diabetes mellitus (GDM) and maternal immune activation (MIA) alter expression of neurodevelopmental and inflammatory genes at gestational day 12.5 (GD12.5). Venn diagrams denote the number of genes increased (a) or decreased (c) in GDM, MIA or GDM+MIA when compared with control mice. The graphics represent gene counts increased (b) or decreased (d) in GDM and GDM+MIA, MIA and GDM+MIA or GDM, MIA and GDM+MIA as indicated. (a, c) Significant changes in gene expression were determined by multiple *t*-tests, and genes with $P < 0.05$, false discovery rate (FDR) < 0.01 were selected. (b, d) Tukey's multiple comparisons two-way analysis of variance (ANOVA) was used to determine significance (** $P < 0.05$) among the groups. Gene symbols are listed according to HUGO Gene Nomenclature Committee.⁷⁵

Figure 5. Differentially expressed transcripts in gestational diabetes mellitus and maternal immune activation (GDM+MIA) condition at gestational day 12.5 (GD12.5). Gene Ontology (GO) enrichment analysis was performed using genes significantly altered in GDM+MIA when compared with control mice fed a normal diet and treated with saline. Clustering was performed using DAVID bioinformatics database analysis ($P < 0.05$).³⁶

Beyond inflammatory mediators, we investigated the extent to which GDM and MIA induced changes in metabolic hormone levels in the maternal serum. Increased serum leptin and insulin levels as well as leptin and insulin insensitivity have been reported in adult mice fed a high-fat diet.^{62,63} In our studies, nonfasting serum insulin levels were significantly affected at GD12.5, regardless of MIA exposure, with no increase noted at GD16.5. In addition, the significant increase in proinflammatory leptin levels observed in GDM increased further with MIA at GD12.5 but

diminished by GD16.5.⁶² Although the effects of GDM on metabolic hormones appeared to remain consistent from GD12.5 to GD16.5, the interaction of MIA and GDM did lessen. We propose that (by a temporary disruption of typical developmental trajectory) this combined acute MIA+GDM insult did leave a lasting consequence on brain development.

Of the transcriptional effects produced by GDM and MIA alone, the hypoxic response caused by both insults is of particular interest. We propose that MIA-induced hypoxia is a consequence

of mounting an antiviral response in the activated fetal brain. In addition, this hypoxia-related expression signature might also reflect an increased oxygen requirement because of a heightened metabolic demand associated with maternal hyperglycemia in GDM.^{64,65} Regardless of the exact mechanism of *Vegfa* and *Flt1* induction, it is likely to result in vascular remodeling that occurs with chronic hypoxia that might persist across the lifespan.^{44,66,67} This finding might have important clinical implications, as microvascular and cerebral blood volume alterations appear to increase risk for developing schizophrenia.^{68,69}

One of the more important questions we sought to answer was whether GDM interacts with MIA to produce a novel phenotype. We did find that MIA differentially affected inflammation and neurodevelopment-associated gene expression in the context of GDM. The altered levels of neurodevelopmental transcripts included crucial patterning genes that are part of neuronal fate differentiation of dopaminergic neurons in the developing midbrain,^{70–72} and altered dopaminergic neuron development has been noted in both GDM and MIA.^{60,73} Our data support that disruption of dopaminergic neuronal development can be strongly exacerbated by a combination of multiple deleterious environmental factors, including MIA and GDM. This process might predispose to increased risk of developing neuropsychiatric disorders in later life and should be investigated in future studies.

In summary, we have shown that combined disruptions to the *in utero* environment interacted to result in an exacerbated transcriptional response in the developing brain. Importantly, neither of these insults (alone or combined) are specific to a particular outcome later in life, but they do predispose to a variety of brain disorders, with diagnosis specificity being genetic context dependent.⁷⁴ More specifically, the ultimate effects of co-occurring GDM and infections during pregnancy most likely depend on the genetic makeup of the developing mother and fetus (or both), likely leading to complex $G_{\text{MOTHER}} \times G_{\text{FETUS}} \times E_{\text{GDM}} \times E_{\text{MIA}}$ interactions that may be critical for determining specific disorder phenotypes. Regardless of this, based on our data we hypothesize that children born to mothers with GDM, exposed to midgestation infections/immune activation, have increased vulnerability to psychiatric disorders later in life, and this could and should be tested in large-scale epidemiologic studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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